A variety of genetic constructs are disclosed that will find both in vitro and in vivo use in the area of tumor biology and cancer therapy. In particular, expression constructs are provided that contain a C-CAM encoding region and other regulatory elements necessary for the expression of a C-CAM transcript. One version of the expression construct is a replication-deficient adenoviral vector. Also provided are methods for the transformation of cell lines and the inhibition of cancer cell proliferation.
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DESCRIPTION

C-CAM EXPRESSION CONSTRUCTS
AND THEIR APPLICATION IN CANCER THERAPY

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates generally to the field of tumor biology. In particular, the invention relates to a nucleic acid encoding a cell adhesion molecule (CAM) and its use in inhibiting tumor growth. In one aspect, the invention relates to expression constructs encoding C-CAM1 and their use in inhibiting prostate and breast cancer.

2. Description of the Related Art

Cancers are one of the leading causes of disease, being responsible for 526,000 deaths in the United States each year (Boring et al., 1993). The leading cancer among U.S. men is prostate carcinoma, a disease of epithelial origin. Almost 50% of patients diagnosed with prostate cancer have already developed metastatic lesions at initial clinical presentation, and an additional 20% of patients who initially have local disease will eventually develop metastasis. The standard regimen for metastatic prostate cancer treatment is by androgen ablation. Despite the initial responsiveness to this treatment, tumor cells inevitably relapse to an androgen-independent (AI) state. The mortality associated with this cancer is mostly due to recurrent AI disease for which effective therapeutic regimens are lacking.
Breast cancer is a major cause of morbidity and mortality in women in the United States, causing about 20% of all cancer deaths in women each year. This disease will develop in about six to seven percent of women in the United States each year, approximately of which half can be cured. Breast cancer is more likely in women who are older, have fibrocystic disease of the breast, have a history of breast cancer in their family, or have had cancer of the endometrium, ovary, or colon. There are several different types of tumors that form in the breast, each with unique characteristics. Most cancers of the breast are detected by the patients themselves, and the average size of the lesion is 2.5 cm. About half of these lesions have already metastasized to the lymph nodes. Early detection by self-examination and mammography followed by biopsy to confirm breast cancer is currently the most effective regimen for diagnosis. The complexity of this malignancy thus offers a significant challenge to conventional cancer therapy strategies. Current treatment protocols include combinations of surgical removal of the cancerous tissue, chemotherapy, radiotherapy, and hormone ablation therapy.

With advances in molecular genetics and biology, it has become evident that altered expression of normal genes leads to initiation of cancer cells. The conventional therapy for malignancy, such as chemotherapy and radiation, has focused on mass cell killing without specific targeting, often resulting in damaging side effects. The new direction in cancer therapy is to deliver a normal gene to replace or correct the mutated gene, thereby altering the malignant phenotype of transformed cells. Several expression constructs have been developed in order to deliver a gene into somatic cells with high efficiency.

Cells can be regulated in a positive (stimulatory) or negative (suppressivemanner. Loss of negative regulation of cell growth is often found in malignant cells which exhibit loss of cell proliferation control. Accumulating molecular genetic evidence has revealed that loss of negative regulators or increase in positive regulators in normal cells will produce such cellular growth abnormalities. Most negative regulators (Marx, 1993; Grunicke and Maly, 1993), referred to as tumor suppressors,
have been found to be involved either in direct control of the cell cycle (e.g., Rb, p53, WT-1) or in the signaling pathway leading to cell growth and differentiation (e.g., NF-1). In addition, recent data suggest that genes related to the maintenance of cell architecture and polarity also may function as tumor suppressors (Marx, 1993; Fearon et al., 1990; Trofatter et al., 1993).

Cell adhesion molecules, or CAM's are known to be involved in a complex network of molecular interactions that regulate organ development and cell differentiation (Edelman, 1985). Recent data indicate that aberrant expression of CAM's maybe involved in the tumorigenesis of several neoplasms; for example, decreased expression of E-cadherin, which is predominantly expressed in epithelial cells, is associated with the progression of several kinds of neoplasms (Edelman and Crossin, 1991; Frixen et al., 1991; Bussemakers et al., 1992; Matsura et al., 1992; Umbas et al., 1992). Also, Giancotti and Ruoslahti (1990) demonstrated that increasing expression of α5β1 integrin by gene transfer can reduce tumorigenicity of Chinese hamster ovary cells in vivo.

C-CAM is expressed in virtually all epithelial cells (Odin and Obrink, 1987). C-CAM, with an apparent molecular weight of 105 kD, was originally isolated from the plasma membrane of the rat hepatocyte by its reaction with specific antibodies that neutralize cell aggregation (Obrink, 1991). Recent studies indicate that, structurally, C-CAM belongs to the immunoglobulin (Ig) superfamily and its sequence is highly homologous to carcinoembryonic antigen (CEA) (Lin and Guidotti, 1989). Using a baculovirus expression system, Cheung et al. (1993a) demonstrated that the first Ig domain of C-CAM is critical for cell adhesive activity.

During liver development, expression of both C-CAM isoforms correlates with differentiation of hepatocytes; the regulation of C-CAM expression is mainly transcriptional (Cheung et al., 1993b; Thompson et al., 1993). Hixson and McEntire (1989) reported an apparent decrease in C-CAM on the cell surface of rat hepatocellular carcinoma lines. In addition, Rosenberg et al. (1993) and Neumaier et al. (1993) demonstrated that mouse and human biliary glycoprotein, a C-CAM
homologue, is down-regulated in colon tumors. Little is known, however, about the role of C-CAM in cancer progression.

3. **Summary of the Invention**

The present invention addresses the need for improved therapy for prostate cancer, breast cancer and other CAM-associated diseases by providing an expression construct containing a nucleic acid encoding a C-CAM. It also is an object of the present invention to provide methods for the use of such compositions and, in particular, use in the treatment of cancer. In another embodiment, the present invention encompasses methods for transforming cells using a C-CAM nucleic acid in an expression construct.

The present invention also encompasses expression constructs that comprises a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, the nucleic acid being under transcriptional control of the promoter.

In a preferred embodiment, the expression constructs further comprise a polyadenylation signal. In another embodiment, the constructs further comprise a selectable marker. In a further embodiment, the expression construct is an adenovirus. In a preferred embodiment, the expression construct is an adenovirus that lacks at least a portion of the E1 region.

In certain embodiments, the nucleic acid is a cDNA. In other embodiments the nucleic acid is a genomic DNA. In an exemplary embodiment the nucleic acid is positioned in a sense orientation with respect to said promoter. In another embodiment the nucleic acid is positioned in an antisense orientation.

The present invention also includes pharmaceutical compositions comprising an expression construct with a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, along with a pharmaceutically acceptable buffer, solvent or diluent. In certain embodiments, the expression construct and pharmaceutically acceptable buffer, solvent or diluent are supplied in a kit.
The invention also provides a method for restoring C-CAM function in a cell that lacks C-CAM function. This method comprises contacting such a cell with an expression construct as described above, wherein the nucleic acid is positioned in a sense orientation. In an exemplary embodiment of the invention, the cell is a transformed cell and the said contacting reverses the transformed phenotype. In a further embodiment, the cell is a prostate tumor cell or a breast tumor cell and, in still a further embodiment, the expression construct is an adenovirus.

The present invention further comprises a method for inhibiting C-CAM function in a cell. This method comprises contacting such a cell with an expression construct as described above, wherein the nucleic acid is positioned in an antisense orientation. In a further embodiment, the expression construct is an adenovirus.

Another embodiment of the invention is a method of treating a mammal with cancer. This method comprises administering to an animal a pharmaceutical composition comprising an expression construct having a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, positioned in a sense orientation, in a pharmaceutically acceptable buffer, solvent or diluent. In a particular embodiment of the invention, the mammal is a human. In another embodiment, administering is via intravenous injection. In a further embodiment, the cancer is prostate, breast or bladder cancer.

In further embodiments the present invention encompasses methods for detecting cancer cells in a sample by detecting a C-CAM or a nucleic acid encoding a C-CAM.

4. **Brief Description of the Drawings**

FIG. 1. **Linear structure of C-CAM.** From the deduced amino acid sequence a linear structure composed of several distinct domains can be predicted. The protein contains two hydrophobic domains, one putative signal sequence in the N-terminal end (indicated by a dashed line) and one transmembrane domain (indicated by a cross-
hatched rectangle) near the C-terminal end. Four immunoglobulin-like domains can be predicted: the most N-terminal one is a V-like domain, the other three are C-2 domains. The C-2 domains, but not the V-like domain, have cysteine residues that could be involved in intradomain disulfide linkages. A consensus sequence for cAMP-dependent phosphorylation (indicated by a “P” in a circle) is found close to the C-terminal end. The protein contains 16 sites for N-linked glycosylation (indicated by a black dot connected to a line). The location of the glycosylation sites suggests that the major N-terminal portion of C-CAM is exposed on the extracellular side, and that the C-terminal end is located on the cytoplasmic side of the plasma membrane.

FIG. 2A. Staining profile of transfected PC-3 cells. PC-3 cells (1 x 10^6) transfected with C-CAM1 cDNA were stained with Ab669 and FITC-conjugated secondary antibodies and subjected to FACS analysis. The dual-parameter histograms show relative cell size (x-axis), as measured by forward light scatter and log of C-CAM1 expression levels (y-axis), as measured by the intensity of green fluorescence emitted by FITC-stained cells. Positive staining was defined as staining intensity greater than 10 FITC units, 10 FITC units being the background level for cells stained with preimmune serum.

FIG. 2B. Staining profile of enriched C-CAM1-positive cell population (cell recovery and cell purity were 93-98% after sorting). PC-3 cells (1 x 10^6) transfected with C-CAM1 cDNA were stained with Ab669 and FITC-conjugated secondary antibodies and subjected to FACS analysis. The dual-parameter histograms show relative cell size (x-axis), as measured by forward light scatter and log of C-CAM1 expression levels (y-axis), as measured by the intensity of green fluorescence emitted by FITC-stained cells. Positive staining was defined as staining intensity greater than 10 FITC units, 10 FITC units being the background level for cells stained with preimmune serum.
FIG. 3. **Adhesion of C-CAM1 Transfected PC-3 Cells**. Single-cell suspensions of PC-3 cells, PC-RSN cells, and C-CAM1 transfected clones were prepared by trypsin digestion of cells from tissue culture plates. One million cells were incubated at room temperature with constant mixing. At the indicated times, the number of single cells was counted with a hemacytometer as described in Materials and Methods, Example II. Legend: PC-3 (open triangles), PC-RSN (closed circles), PC-L1 (open circles), PC-L2 (closed triangles), PC-L6 (closed square), PC-L9 (open square).

FIG. 4A. **In vitro growth rate of C-CAM1-transfected PC-3 clones as determined by crystal violet staining**. Five thousand cells were plated on a 24-well plate in the presence of T medium containing 1% FBS. The medium was changed every 4 days, and the number of cells was determined every 48 hr by the crystal violet assay. Legend: PC-3 (closed circles), PC-RSN (open triangles), PC-L1 (open circles), PC-L2 (closed triangles), PC-L6 (closed square), PC-L9 (open square).

FIG. 4B. **In vitro growth rate of C-CAM1-transfected PC-3 clones as determined by [³H]thymidine incorporation**. Five thousand cells were plated as in FIG. 4A. Fresh medium containing 0.1 mCi of [³H]thymidine was added and, after 2 hr, the cells were harvested and the amount of [³H]thymidine incorporated into the cells was determined. Columns = mean; bars = SD.

FIG. 5A. **Study 1 - Change in Tumor Volume of C-CAM1-Transfected PC-3 Cells In vivo**. Each animal was injected subcutaneously at six sites. The volume of each tumor was recorded weekly when tumors became palpable. Legend: PC-3 (closed circles), PC-RSN (open triangles), PC-L1 (open circles), PC-L2 (closed triangles).
FIG. 5B. Study 2 - Change in Tumor Volume of C-CAM1-Transfected PC-3 Cells In vivo. Each animal was injected subcutaneously at six sites. The volume of each tumor was recorded weekly when tumors became palpable. Legend: PC-RSN (open triangles), PC-L6 (closed square), PC-L9 (open square).

FIG. 6. C-CAM1 recombinant adenovirus construction. A full-length C-CAM1 cDNA (2.1 kB) was first cloned into the EcoRI site of the pBSK vector with two possible orientations (sense and antisense). Subsequently, either strand of C-CAM1 insert was directional cloned into the HindIII (5') and NotI (3') sites of pAdE1CMV/pA under the control of a CMV promoter. pJM17 vector contains both viral genome and pBR322 sequences indicated by triangle. Plaques appeared between day 8 and day 10. Each clone of adenovirus was subjected to structural analysis. mu = map unit.

FIG. 7. Determination of the genome structure of C-CAM1 adenovirus by PCR. The AdCAM genome of 36 kB is divided into 100 map units (1 mu = 0.36 kB). The C-CAM expression cassette replaced the E1 region (1.3-9.2 mu) of the Ad5 genome. To confirm the structure of AdCAM, three different sets of primers were used. Primer set A is designed to amplify a 300-bp transcript from the C-terminus of C-CAM1 cDNA. Primer set B, including the forward primer from the first intron downstream of human CMV major IE gene promoter and reverse primer from SV40 early polyadenylation signal, is designed to amplify 2.1-kb of the cDNA insert. Primer set C, with two primers localized at 11 and 13.4 mu, is designed to amplify the Ad5 genome-specific sequences (~0.86-kb PCR™ product). mu = map unit.

FIG. 8A. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with PBS as a control for 1 day. At the indicated times, the percentage of infected cells was determined using immunofluorescence staining by fluorescent-activated cell scanning analysis. The dual-parameter
histogram represents relative cell size (x-axis) measured from forward light scatters (FSC) and log of C-CAM1 expression levels (y-axis) measured from the green fluorescence intensity emitted by FITC-stained cells. Positive staining was defined as staining intensity greater than 20 FITC units, which was the background level from control cells as well as the levels from AdCAM101-infected cells stained with antibodies. Fraction of C-CAM-positive cells = 2%.

FIG. 8B. **Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry.** Cells were infected with AdCAM101 (1,000:1 virions:cell) for 1 day. Fractions of C-CAM-positive cells = 1%.

FIG. 8C. **Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry.** Cells were infected with AdCAM902 (100:1 virions:cell) for 1 day. Fractions of C-CAM-positive cells = 48%.

FIG. 8D. **Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry.** Cells were infected with AdCAM902 1,000:1 virions:cell) for 1 day. Fractions of C-CAM-positive cells = 93%.

FIG. 8E. **Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry.** Cells were infected with PBS as a control for 2 days. Fractions of C-CAM-positive cells = 4%.

FIG. 8F. **Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry.** Cells were infected with AdCAM101 (1,000:1 virions:cell) for 2 days. Fractions of C-CAM-positive cells = 6%.
FIG. 8G. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM902 (100:1 virions:cell) for 2 days. Fractions of C-CAM-positive cells = 62%.

FIG. 8H. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM902 1,000:1 virions:cell) for 2 days. Fractions of C-CAM-positive cells = 92%.

FIG. 8I. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with PBS as a control for 4 days. Fractions of C-CAM-positive cells = 2%.

FIG. 8J. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM101 (1,000:1 virions:cell) for 4 days. Fractions of C-CAM-positive cells = 2%.

FIG. 8K. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM902 (100:1 virions:cell) for 4 days. Fractions of C-CAM-positive cells = 77%.

FIG. 8L. Efficiency of infection by adenovirus in PC-3 cells as determined by flow cytometry. Cells were infected with AdCAM902 1,000:1 virions:cell) for 4 days. Fractions of C-CAM-positive cells = 95%.

FIG. 9. The tumor-suppressing effect of C-CAM1 adenovirus on PC-3 tumors. Fourteen tumors were treated in each group. A single dose of 50 ml of either control (PBS + 10% glycerol) or viral solution (5x10^{11} AdCAM101 or AdCAM902 virions/ml in PBS + 10% glycerol) was injected subcutaneously above the tumor mass 20 days after inoculation with PC-3 cells. Tumor volume was recorded every week
and tabulated as mean± standard deviation (indicated by error bars). Arrow indicates viral injection. Legend: control (closed triangles), AdCAM101 (open circles), AdCAM902 (closed circles).

FIG. 10A. Control - Determination of the treatment schedule of C-CAM1 adenovirus for PC-3 tumors. Five tumors (each indicated by a different symbol) were treated in each group. Control: a dose of 50 ml of PBS + 10% glycerol was injected once a week for three consecutive weeks when tumor volume was larger than 40 mm³. Tumor volume was recorded every week. Arrow indicates viral injection.

FIG. 10B. AdCAM101 - Determination of the treatment schedule of C-CAM1 adenovirus for PC-3 tumors. Five tumors (each indicated by a different symbol) were treated in each group. 5x10¹¹ AdCAM101 virions/ml in PBS + 10% glycerol was injected once a week for three consecutive weeks when tumor volume was larger than 40 mm³. Tumor volume was recorded every week. Arrow indicates viral injection.

FIG. 10C. AdCAM902 - Determination of the treatment schedule of C-CAM1 adenovirus for PC-3 tumors. Five tumors (each indicated by a different symbol) were treated in each group. A dose of 5x10¹¹ AdCAM902 virions/ml in PBS + 10% glycerol was injected once a week for three consecutive weeks when tumor volume was larger than 40 mm³. Tumor volume was recorded every week. Arrow indicates viral injection.

FIG. 11. Structure of wild type and mutant C-CAM proteins. The Ig domains are labeled D1 to D4. sig, signal sequence; L, linker domain; TM, transmembrane domain; cyto, cytoplasmic domain.

FIG. 12A. Controls - Effects of C-CAM1 and its mutants on the tumorigenicity of MDA-MB-468 cells in vivo. MDA-MB-468 cells were uninfected,
infected with AdCAM1-AS, or infected with AdCAM1. Twenty-four hours after infection, the cells were injected subcutaneously into nude mice at $10^6$ cells per site. Each column represents one tumor site. The tumor sizes were recorded at four weeks.

FIG. 12B. Mutants - Effects of C-CAM1 and its mutants on the tumorigenicity of MDA-MB-468 cells in vivo. MDA-MB-468 cells were infected with AdCAM1-ΔD1, AdCAM1-H458 or AdCAM1-G454. Twenty-four hours after infection, the cells were injected subcutaneously into nude mice at $10^6$ cells per site. Each column represents one tumor site. The tumor sizes were recorded at four weeks.

FIG. 13. Aggregation of MDA-MB-468 cells expressing C-CAM1 mutants. MDA-MB-468 cells were infected with AdCAM1 or the mutants indicated, and the cell adhesion assay was performed as described in Example IX. The aggregation of cells is expressed as the decrease in the percentage of single cells.

5. Detailed Description of the Preferred Embodiments

The present inventors now have determined that C-CAM can act as a tumor suppressor. In light of this observation, there are at least two uses for C-CAM-related nucleic acids and constructs for their expression. First, in the context of cell culture, it may be possible to generate C-CAM negative cells by targeting endogenous C-CAM with antisense methodology in order to immortalize such cells. This may prove particularly interesting in the generation of cell lines for the study of cancer and, in particular, prostate and breast cancer. Second, the present invention addresses the need for improved therapy for cancer and other C-CAM-associated diseases. In particular, an expression construct capable of expressing a functional C-CAM product can be used to inhibit tumor cell proliferation. The following description provides a more detailed explanation of these and other aspects of the present invention.
C-CAM AND C-CAM-RELATED NUCLEIC ACIDS

A. C-CAM

The nucleic acid of the present invention may encode an entire C-CAM gene, a functional C-CAM protein domain, or any C-CAM polypeptide, peptide or fragment that is sufficient to affect cell adhesion. The C-CAM nucleic acid may be derived from genomic DNA, i.e., cloned directly from the genome of a particular organism. In preferred embodiments, however, the nucleic acid encoding C-CAM would comprise of complementary DNA (cDNA). The term "cDNA" used here, is intended to refer to DNA prepared using messenger RNA (mRNA) as template. The advantage of using a cDNA, as opposed to genomic DNA or DNA polymerized from a genomic DNA or non- or partially-processed RNA template, is that the cDNA does not contain any non-coding sequences but, rather, contains only the coding region of the corresponding protein. There may be times when the full or partial genomic sequence is preferred, however, such as where the non-coding regions are required for optimal expression or where non-coding regions such as introns are to be targeted in an antisense strategy. Throughout the application, the term C-CAM is intended to refer to the rat C-CAM or cell-CAM 105, the human C-CAM homologue (biliary glycoprotein 1 (BGP1)), and all C-CAM homologues from other species.

(i) C-CAM Protein Structure

C-CAM, like all other known cell adhesion molecules (CAMs), are large intrinsic cell-surface glycoproteins that are mobile in the plane of the membrane (Gall and Edeleman 1981; Pollerberg et al., 1986). CAMs mediate cell recognition and adhesion and are of prime importance for the formation and integrity of tissues. The majority of the known CAMs belongs to one of the following families: the immunoglobulin (Ig) superfamily (Williams et al., 1988), the cadherin family (Takeichi, 1988), the integrin superfamily (Hynes, 1987), the LEC-CAM family (Stoolman, 1989) and the H-CAM family (Stoolman, 1989). The cell adhesion
molecule C-CAM belongs to the immunoglobulin superfamily, and more specifically is a member of the carcinoembryonic antigen (CEA) gene family.

The Ig-superfamily is a large family that, in addition to the immunoglobulins, contains many other proteins, the majority being involved in cellular recognition phenomena (Williams et al., 1988). The common building block is the immunoglobulin domain of about 100 amino acid residues that is arranged as a sandwich of two sheets of anti-parallel \( \beta \)-strands. Different members of the superfamily have varying numbers of Ig-like domains. A list of putative CAMs presently known, that belong to the Ig-superfamily, is given in Table 1. One characteristic feature of CAMs in the Ig-superfamily is that they are calcium-independent, that is, they do not need calcium ions for their binding activity. Both homophilic and heterophilic binding occur among the members of this family.

There seem to be several subfamilies of CAMs in the Ig-superfamily. One of these is the carcinoembryonic antigen (CEA) family (Benchimol et al., 1989), which contains a large number of different proteins that can be either transmembrane, GPI-linked, or secreted (Khan et al., 1989). Several of the members of the CEA family cross-react immunologically. It has been reported that both CEA itself, which has seven Ig-domains, and NCA (nonspecific cross-reactive antigen), which has three Ig-domains can function both as homophilic and heterophilic, calcium-independent cell adhesion molecules (Benchimol et al., 1989; Oikawa et al., 1989).

A close examination of the amino acid sequence of rat C-CAM reveals that it is a member of the immunoglobulin superfamily (Aurivillius et al., 1990). Lin and Guidotti noted that the ecto-ATPase/C-CAM is highly homologous to human biliary glycoprotein 1 (BGP1), with 65% of the amino acids being identical (Lin et al., 1989). BGP1 is a member of the CEA-family (Aurivillius et al., 1990; Lin et al., 1989). Thus, it has been concluded that C-CAM belongs to the CEA-subfamily of the immunoglobulin superfamily.

So far, two C-CAM isoforms, C-CAM1 and C-CAM2, have been identified. Both isoforms are composed of a cytoplasmic domain, a 25 amino acid long
transmembrane domain, and an extracellular domain with four Ig-like domains (FIG. 1). Sixteen potential sites for N-glycosylation are found in the extracellular domain, a finding that agrees well with the chemical determination of the carbohydrate content in the mature protein (Obrink 1991). C-CAM1 and C-CAM2 differ in their first Ig domains by 16 amino acids and in the length of their cytoplasmic domains (Culic et al., 1992). C-CAM1, but not C-CAM2, shows adhesion activity when expressed in a baculoviral vector in insect cells (Cheung et al., 1993a). C-CAM binds to itself in a homophilic manner. Further structural and functional analyses of C-CAM1 by Cheung et al. (1993a) indicated that the presence of the first Ig domain is essential for adhesion.

<table>
<thead>
<tr>
<th>CAM</th>
<th>Cell Type</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-CAM</td>
<td>Neuron, glia muscle</td>
<td>Mammals, birds, amphibians</td>
<td>Williams et al., 1988, Hansson et al., 1990</td>
</tr>
<tr>
<td>L-1 Ng-CAM</td>
<td>Neuron Ep Gila</td>
<td>Mouse, rat, chicken, Man, mouse, rat, chicken, cow</td>
<td>Moos et al., 1988, Salzer et al., 1987</td>
</tr>
<tr>
<td>L1E G4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Po</td>
<td>Schwann</td>
<td>Rat</td>
<td>Lemke et al., 1988</td>
</tr>
<tr>
<td>Contactin</td>
<td>Neuron</td>
<td>Chicken</td>
<td>Ranscht, 1988</td>
</tr>
<tr>
<td>F11</td>
<td>Neuron</td>
<td>Chicken</td>
<td>Brümmendorf et al., 1989</td>
</tr>
<tr>
<td>F3</td>
<td>Neuron</td>
<td>Mouse</td>
<td>Gennarini et al., 1989</td>
</tr>
<tr>
<td>Fasciclin II</td>
<td>Neuron</td>
<td>Grasshopper</td>
<td>Harrelson et al., 1988</td>
</tr>
<tr>
<td>Neuroglian</td>
<td>Neuron, glia, many other cell types</td>
<td>Drosophila</td>
<td>Bieber et al., 1989</td>
</tr>
<tr>
<td>Amalgam</td>
<td>Neuron</td>
<td>Drosophila</td>
<td>Seeger et al., 1988</td>
</tr>
<tr>
<td>TAG-1</td>
<td>Neuron</td>
<td>Rat</td>
<td>Furley et al., 1990</td>
</tr>
<tr>
<td>C-CAM</td>
<td>Ep. En. Lc.</td>
<td>Rat, man</td>
<td>Odin et al., 1988; Tingstrom et al., 1990; Aurivillius et al., 1990</td>
</tr>
</tbody>
</table>
Table 1. CAMs in the Ig-superfamily (continued)

<table>
<thead>
<tr>
<th>CAM</th>
<th>Cell Type</th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEA</td>
<td>Ep.</td>
<td>Man</td>
<td>Benchimol et al., 1989</td>
</tr>
<tr>
<td>NCA</td>
<td>Ep. Lc.</td>
<td>Man</td>
<td>Oikawa et al., 1989</td>
</tr>
<tr>
<td>DCC</td>
<td>Ep.</td>
<td>Man</td>
<td>Fearoz et al., 1990</td>
</tr>
<tr>
<td>GP42</td>
<td>Fibroblast</td>
<td>Mouse</td>
<td>Alturda et al., 1989</td>
</tr>
<tr>
<td>MUC18</td>
<td>Melanoma</td>
<td>Man</td>
<td>Lehmann et al., 1989</td>
</tr>
<tr>
<td>PECAM1/End</td>
<td>Lc. En. 5M. Trc</td>
<td>Man, cow</td>
<td>Newman et al., 1990</td>
</tr>
<tr>
<td>o-CAM/CD31</td>
<td>En</td>
<td>Man</td>
<td>Elices et al., 1990</td>
</tr>
<tr>
<td>VCAM1/</td>
<td>Lc. many cell</td>
<td>Man, mouse</td>
<td>Staunton et al., 1988</td>
</tr>
<tr>
<td>INCAM-110</td>
<td>types</td>
<td>Man</td>
<td>Staunton et al., 1989</td>
</tr>
<tr>
<td>ICAM1/CD54</td>
<td>En</td>
<td>Man</td>
<td>Streuli et al., 1988</td>
</tr>
<tr>
<td>ICAM-2</td>
<td>Ly. kidney,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAR</td>
<td>prostate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD2</td>
<td>Ly</td>
<td>Man</td>
<td>Williams and Barclay, 1988</td>
</tr>
<tr>
<td>LFA-3/CD58</td>
<td>Many cell types</td>
<td>Man</td>
<td>Williams and Barclay, 1988</td>
</tr>
<tr>
<td>CD4</td>
<td>Ly</td>
<td>Man, mouse</td>
<td>Williams and Barclay, 1988</td>
</tr>
<tr>
<td>CD8</td>
<td>Ly</td>
<td>Man, mouse</td>
<td>Williams and Barclay, 1988</td>
</tr>
<tr>
<td>MHC I</td>
<td>All kinds</td>
<td>Man, mouse, etc.</td>
<td>Williams and Barclay, 1988</td>
</tr>
<tr>
<td>MHC II</td>
<td>Mo. dendritic</td>
<td>Man, mouse, etc.</td>
<td>Williams and Barclay, 1988</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cells</td>
<td>Man, mouse, etc.</td>
<td>Williams and Barclay, 1988</td>
</tr>
<tr>
<td>CD28</td>
<td>T-cells</td>
<td>Man</td>
<td>Linsley et al., 1990</td>
</tr>
<tr>
<td>B7/BB-1</td>
<td>B-cells</td>
<td>Man, mouse</td>
<td>Linsley et al., 1990</td>
</tr>
<tr>
<td>B29</td>
<td>B-cells</td>
<td>Mouse</td>
<td>Hermanson et al., 1988</td>
</tr>
</tbody>
</table>

(ii) C-CAM Location and Function

C-CAM antigens are expressed in a number of organs of mature rats (Odin et al., 1988). The tissue distribution within these organs is, however, limited. Thus, C-CAM is primarily expressed in various epithelia (including hepatocytes of the liver), endothelia of capillaries, small arteries and veins, and in megakaryocytes, platelets,
polymorphonuclear leukocytes and a subset of mononuclear leukocytes (Odin et al., 1988). It has not been found in nervous tissues, muscle tissues or connective tissues. C-CAM appears rather late in the fetal development of the liver and goes through transient down-regulation during regeneration after partial heptectomy (Odin et al., 1986). In this context, it is also interesting to note that the expression of C-CAM is significantly altered in hepatocellular carcinomas, so that it is either missing or chemically modified (Hinson et al., 1985; Hinson et al., 1989). In addition, Rosenberg et al. (1993) and Neumaier et al. (1993) demonstrated that mouse and human biliary glycoprotein, a C-CAM homologue, is down-regulated in colon tumors.

Odin et al. (1988) using highly sensitive immunodetection systems were not able to detect C-CAM in the prostate gland nor the epithelium of the urinary bladder. Yet the inventors have not only detected C-CAM in prostate and bladder cells but also identified the role of C-CAM in in these cells as a tumor cell supressor. C-CAM has not been detected in breast cancer cells, yet it is able to act as a tumor suppressor, as disclosed in Example X. The urinary bladder is a musculomembranous sac, situated in the anterior part of the pelvic cavity, that serves as a reservoir for urine which it recieves through the ureters and discharges through the urethra. While the prostate is a male gland that surrounds the neck of the bladder and the urethra.

Example V, disclosed herein, demonstrated that C-CAM was associated with basal cells from the normal prostate gland obtained from fetal, juvenile and adult prostate. In contrast, there is a complete disappearance of C-CAM in prostate carcinoma, suggesting that down-regulation of C-CAM is associated with the onset of hyperplastic/neoplastic transformation. Examples I through IV, demonstrated that expression of C-CAM suppresses the tumorigenicity of a prostatic cancer cell line, PC-3. Also, using an antisense strategy, endogenous C-CAM expression in a nontumorigenic prostatic epithelial cell line (NbE) was reduced, thereby inducing tumor formation by these cells. In Examples VI and VII, the delivery of C-CAM adenovirus was able to repress the growth of PC-3 induced tumors in mice. Finally,
Example X demonstrated that expression of C-CAM1 suppresses the *in vivo* growth of a breast cancer cell line, MDA-MB-468.

Although a preferred embodiment of the invention is the inhibition of tumor proliferation, transformation and detection of cancer in prostate, breast and bladder cells, any cell or tissue which is affected by C-CAM expression is within the scope of this invention.

(iii) C-CAM Mechanism of Action

Although the mechanisms by which C-CAM affects these processes are not clear, these cell-surface adhesion molecules most likely exert their biological effects through interactions with other cellular molecules, that transduce outside signals into the cells. For example, the integrins, which are Ca\(^{2+}\)-dependent CAMs known to interact with a variety of extracellular matrices, have been shown to associate with several intracellular tyrosine kinases (Luna and Hitt, 1992). On the other hand, CAMs have also been shown to participate in the organization of cell architecture by connecting cytoskeleton molecules through an adherens-junction protein complex. Recent molecular genetic analyses (Trofatter *et al.*, 1993) suggest that a potential tumor suppressor gene (NF-2) involved in neurofibromatosis belongs to the family of adherens junction molecules. Also, in the case of E-cadherin, at least three cytoplasmic proteins, \(\alpha\)-, \(\beta\)-, and \(\gamma\)-catenin, are associated with the cytoplasmic domain of cadherin (Takeichi, 1991). These associated proteins, which are part of the adherens junction proteins, play important roles in maintaining cellular architecture (Geiger, 1983). Consistent with this observation, recently it was found that \(\alpha\)- and \(\beta\)-catenin were also associated with the product of a potential tumor suppressor gene involved in colon carcinogenesis, APC (Rubinfeld *et al.*, 1993; Su *et al.*, 1993). These observations suggest that a cell-adhesion molecule can regulate cell growth through indirect interactions with a tumor suppressor.

The cytoplasmic domain of C-CAM1 contains several potential phosphorylation sites, including one for cAMP-dependent kinase and one for tyrosine
kinase. The putative tyrosine phosphorylation sequence is also within the consensus sequence for the antigen-receptor homology domain (Cambier and Campbell, 1992) that is postulated to be important for signal transduction from membrane-bound IgM molecules in B cells. These structural features suggest that the cytoplasmic domain of C-CAM1 may be critical for signal transduction and that C-CAM1 may interact with other kinases to elicit a negative signal for cell growth. Examples IX and X, disclosed herein, demonstrated that the cytoplasmic domain of C-CAM1 is involved in suppressing growth of the breast tumor cell line MDA-MB-468, and that adhesion does not appear to be required for tumor suppression. It is also possible that the cytoplasmic domain of C-CAM1 interacts with cytoskeletal proteins that modulate cellular morphology.

(iv) C-CAM and Cell Adhesion

While nucleic acid encoding a C-CAM1 (as disclosed herein as SEQ ID NO:1) is the preferred embodiment of the invention, it is believed that nucleic acid encoding C-CAM-related proteins will also function according to the present invention. For example, in certain embodiments, nucleic acids encoding any one of the domains of C-CAM, as seen in FIG. 1 and described above, may function according to the present invention. Thus, one embodiment of the invention may comprise a nucleic acid encoding all or part of the cytoplasmic domain, with or without phosphorylation sites. In another embodiment of the invention, a nucleic acid may encode for all, part or none of the transmembrane portion of C-CAM. In a further embodiment, a nucleic acid may encode for part or all of the extracellular domain, with or without sites for N-glycosylation. In yet a further embodiment, a nucleic acid encoding a domain or peptide from another CAM, such as a member of the Ig superfamily listed in Table 1, may be included in the present invention. It is envisioned that, without undue experimentation, a nucleic acid encoding a CAM may be obtained comprising of several different CAM domains or peptides and still function according to the present invention.
It is also envisioned that a nucleic acid encoding a C-CAM or a related C-CAM may have variations within its sequences that are biological functional equivalents (see below). In order to function according to the present invention the nucleic acid encoding a C-CAM must affect cell adhesion. To test for such an affect, it is a simple matter to assay binding of a protein, encoded by a C-CAM nucleic acid, \textit{in vitro} or by the use of transfection techniques as described in the Examples and disclosed herein.

\textit{In vitro} binding assays have been performed on N-CAM and Ng-CAM linked to Covaspheres or other solid substrates, or in artificial lipid vesicles (Hoffman and Edelman, 1983; Sadoul et al., 1983; Grumet and Edelman, 1988) to determine CAM binding kinetics and mechanisms. These methods also may be employed, alone or in conjunction with other methods, to determine the affect of related C-CAMs on adhesion.

Preferred methods for testing the affect a nucleic acid encoding a C-CAM has on cell adhesion properties, include the use of transfection studies. Methods for transfecting cells \textit{in vitro} and for assaying cell adhesion and anchorage-independent growth are described in detail in Examples I through III, and IX, while assessment of \textit{in vivo} tumorigenicity is described in Examples III, VI, VII and X.

(v) C-CAM Biological Functional Equivalents

As used in this application, the term "nucleic acid encoding a C-CAM" refers to a nucleic acid molecule that has been isolated free of total cellular nucleic acid. In preferred embodiments, the invention concerns a nucleic acid sequence essentially as set forth in SEQ ID NO:1. The term "as set forth in SEQ ID NO:1" means that the nucleic acid sequence substantially corresponds to a portion of SEQ ID NO:1 and has relatively few codons that are not identical, or functionally equivalent, to the codons of SEQ ID NO:1. The term "functionally equivalent codon" is used herein to refer to codons that encode the same amino acid, such as the six codons for arginine or serine,
and also refers to codons that encode biologically equivalent amino acids (as in Table 2 below).

Allowing for the degeneracy of the genetic code, sequences that have between about 50% and about 75%; or more preferably, between about 76% and about 99% of nucleotides that are identical to the nucleotides of SEQ ID NO:1 will be sequences that are "as set forth in SEQ ID NO:1". Sequences that are essentially the same as those set forth in SEQ ID NO:1 may also be functionally defined as sequences that are capable of hybridizing to a nucleic acid segment containing the complement of SEQ ID NO:1 under standard conditions. Suitable hybridization conditions will be well known to those of skill in the art and are clearly set forth herein, e.g., see Example I.
<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Codons</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala A</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys C</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>Asp D</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>Glu E</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe F</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly G</td>
</tr>
<tr>
<td>Histidine</td>
<td>His H</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Ile I</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys K</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leu L</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met M</td>
</tr>
<tr>
<td>Asparagine</td>
<td>Asn N</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro P</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln Q</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg R</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser S</td>
</tr>
<tr>
<td>Threonine</td>
<td>Thr T</td>
</tr>
<tr>
<td>Valine</td>
<td>Val V</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp W</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Tyr Y</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Codons</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCA GCC GCG GCU</td>
</tr>
<tr>
<td>UGC UGU</td>
</tr>
<tr>
<td>GAC GAU</td>
</tr>
<tr>
<td>GAA GAG</td>
</tr>
<tr>
<td>UUC UUU</td>
</tr>
<tr>
<td>GGA GGC GGG GU</td>
</tr>
<tr>
<td>CAC CAU</td>
</tr>
<tr>
<td>AUA AUC AUU</td>
</tr>
<tr>
<td>AAA AAG</td>
</tr>
<tr>
<td>UUA UUG CUA CUC CUG CUU</td>
</tr>
<tr>
<td>AUG</td>
</tr>
<tr>
<td>AAC AAU</td>
</tr>
<tr>
<td>CCA CCC CCG CCU</td>
</tr>
<tr>
<td>CAA CAG</td>
</tr>
<tr>
<td>AGA AGG CGA CGC CGG CGU</td>
</tr>
<tr>
<td>AGC AGU UCA UCC UCG UCU</td>
</tr>
<tr>
<td>ACA ACC ACG ACU</td>
</tr>
<tr>
<td>GUA GUC GUG GUU</td>
</tr>
<tr>
<td>UAC UAU</td>
</tr>
</tbody>
</table>
Naturally, the present invention also encompasses DNA segments that are complementary, or essentially complementary, to the sequence set forth in SEQ ID NO:1. Nucleic acid sequences that are "complementarily" are those that are capable of base-pairing according to the standard Watson-Crick complementary rules. As used herein, the term "complementary sequences" means nucleic acid sequences that are substantially complementary, as may be assessed by the same nucleotide comparison set forth above, or as defined as being capable of hybridizing to the nucleic acid segment of SEQ ID NO:1 under relatively stringent conditions such as those described herein.

The DNA segments of the present invention include those encoding biologically functional equivalent C-CAM proteins and peptides. Such sequences may arise as a consequence of codon redundancy and functional equivalency that are known to occur naturally within nucleic acid sequences and the proteins thus encoded. Alternatively, functionally equivalent proteins or peptides may be created via the application of recombinant DNA technology, in which changes in the protein structure may be engineered, based on considerations of the properties of the amino acids being exchanged. Changes designed by man may be introduced through the application of site-directed mutagenesis techniques, e.g., to introduce improvements to the adhesiveness of the protein or to test mutants in order to examine signal transducing activity at the molecular level.

If desired, one may also prepare fusion proteins and peptides, e.g., where the C-CAM coding regions are aligned within the same expression unit with other proteins or peptides having desired functions, such as for purification or immunodetection purposes (e.g., proteins that may be purified by affinity chromatography and enzyme label coding regions, respectively).
It will also be understood that amino acid and nucleic acid sequences may include additional residues, such as additional N-or C-terminal amino acids or 5' or 3' sequences, and yet still be essentially as set forth in one of the sequences disclosed herein, so long as the sequence meets the criteria set forth above, including the maintenance of biological protein activity where protein expression is concerned. The addition of terminal sequences particularly applies to coding nucleic acid sequences that may, for example, include various non-coding sequences flanking either of the 5' or 3' portions of the coding region, such as promoters.

As mentioned above, modification and changes may be made in the structure of C-CAM (as exemplified by SEQ ID NO:2) and still obtain a molecule having like or otherwise desirable characteristics. For example, certain amino acids may be substituted for other amino acids in a protein structure without appreciable loss of interactive binding capacity with structures such as, for example, antigen-binding regions of antibodies or binding sites on substrate molecules, receptors, or signal transduction. Since it is the interactive capacity and nature of a protein that defines that protein's biological functional activity, certain amino acid sequence substitutions can be made in a protein sequence (or, of course, its underlying DNA coding sequence) and nevertheless obtain a protein with like (agonistic) properties. Equally, the same considerations may be employed to create a protein or polypeptide with countervailing (e.g., antagonistic) properties. It is thus contemplated by the inventors that various changes may be made in the sequence of C-CAM proteins or peptides (or underlying DNA) without appreciable loss of their biological utility or activity.
It is also well understood by the skilled artisan that, inherent in the definition of a biologically functional equivalent protein or peptide, is the concept that there is a limit to the number of changes that may be made within a defined portion of the molecule and still result in a molecule with an acceptable level of equivalent biological activity. Biologically functional equivalent peptides are thus defined herein as those peptides in which certain, not most or all, of the amino acids may be substituted. In particular, where the N-terminal of the C-CAM protein is concerned, it is contemplated that only about 16 or more preferably, about 5 amino acids may be changed within a given peptide. Of course, a plurality of distinct proteins/peptides with different substitutions may easily be made and used in accordance with the invention.

Amino acid substitutions are generally based on the relative similarity of the amino acid side-chain substituents, for example, their hydrophobicity, hydrophilicity, charge, size, and the like. An analysis of the size, shape and type of the amino acid side-chain substituents reveals that arginine, lysine and histidine are all positively charged residues; that alanine, glycine and serine are all a similar size; and that phenylalanine, tryptophan and tyrosine all have a generally similar shape. Therefore, based upon these considerations, arginine, lysine and histidine; alanine, glycine and serine; and phenylalanine, tryptophan and tyrosine; are defined herein as biologically functional equivalents.

In making changes, the hydrophobic index of amino acids may be considered. Each amino acid has been assigned a hydrophobic index on the basis of their hydrophobicity and charge characteristics, these are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5).
The importance of the hydrophobic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte & Doolittle, 1982, incorporated herein by reference). It is known that certain amino acids may be substituted for other amino acids having a similar hydrophobic index or score and still retain a similar biological activity. In making changes based upon the hydrophobic index, the substitution of amino acids whose hydrophobic indices are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

It is understood that an amino acid can be substituted for another having a similar hydrophilicity value and still obtain a biologically equivalent protein. As detailed in U.S. Patent 4,554,101, the following hydrophilicity values have been assigned to amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 ± 1); glutamate (+3.0 ± 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 ± 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); tryptophan (-3.4).

In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ±2 is preferred, those which are within ±1 are particularly preferred, and those within ±0.5 are even more particularly preferred.

While the preceding discussion has focused on functionally equivalent polypeptides arising from amino acid changes, it will be appreciated that these changes may be effected by alteration of the encoding DNA, taking into consideration also that the genetic code is degenerate and that two or more codons may code for the same amino acid.
In addition to the peptidyl compounds described herein, the inventors also contemplate that other sterically similar compounds may be formulated to mimic the key portions of the peptide structure. Such compounds, which may be termed peptidomimetics, may be used in the same manner as the peptides of the invention and hence are also functional equivalents. The generation of a structural functional equivalent may be achieved by the techniques of modelling and chemical design known to those of skill in the art. It will be understood that all such sterically similar constructs fall within the scope of the present invention.

B. Antisense Constructs

In an alternative embodiment, the C-CAM nucleic acid may encode the antisense version of any of the above full-length or fragmentary nucleic acids. The sense or coding constructs will generally be used in methods for inhibiting tumor proliferation where the lack of CAM function is a problem and replacement of CAM function is desired. However, in embodiments where overexpression of CAM is a problem, such as where inhibition or suppression of CAM expression is desired, antisense molecules may be employed.

The term "antisense nucleic acid" is intended to refer to the oligonucleotides complementary to the base sequences of CAM-encoding DNA and RNA. Antisense oligonucleotides, when introduced into a target cell, specifically bind to their target nucleic acid and interfere with transcription, RNA processing, transport and/or translation. Targeting double-stranded (ds) DNA with oligonucleotide leads to triple-helix formation; targeting RNA will lead to double-helix formation.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription or translation or both within a host cell, either in vitro or in vivo, such as within a host animal, including a human subject. Nucleic acid sequences which comprising "complementary nucleotides" are those which are capable of base-
pairing according to the standard Watson-Crick complementarity rules. That is, that the larger purines will base pair with the smaller pyrimidines to form only combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T), in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA.

As used herein, the terms "complementary" or "antisense sequences" mean nucleic acid sequences that are substantially complementary over their entire length and have very few base mismatches. For example, nucleic acid sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen positions with only a single mismatch. Naturally, nucleic acid sequences which are "completely complementary" will be nucleic acid sequences which are entirely complementary throughout their entire length and have no base mismatches.

While all or part of the gene sequence may be employed in the context of antisense construction, statistically, any sequence of 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase in vivo accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs will be used. One can readily determine whether a given antisense nucleic acid is effective at targeting of the corresponding host cell gene simply by testing the constructs in vitro to determine whether the endogenous gene's function is affected or whether the expression of related genes having complementary sequences is affected.

In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have
been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner et al., 1993).

As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" is refers to the an RNA-based enzyme capable targeting cleaving particular base sequences in C-CAM DNA and RNA. Ribozymes can either be targeted directly to cells, in the form of RNA oligo-nucleotides incorporating ribozyme sequences, or introduced into the cell as an expression construct encoding the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense nucleic acids.

EXPRESSION CONSTRUCTS
Throughout this application, the term "expression construct" is meant to include any type of genetic construct containing a nucleic acid coding for a gene product in which part or all of the nucleic acid encoding sequence is capable of being transcribed. The transcript may be translated into a protein, but it need not be. Thus, in certain embodiments, expression includes both transcription of a CAM gene and translation of a CAM mRNA into a CAM gene product. In other embodiments, expression only includes transcription of the nucleic acid encoding a CAM.

A. Promoters and Enhancers
In preferred embodiments, the nucleic acid encoding a CAM is under transcriptional control of a promoter. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the cell, required to initiate the specific transcription of a gene. The phrase "under transcriptional control" means that the promoter is in the correct location and orientation in relation to the nucleic acid to control RNA polymerase initiation and expression of the gene.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II. Much of the thinking about how promoters are organized derives from analyses of
several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for transcriptional activator proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between elements is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either co-operatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a nucleic acid encoding a CAM is not believed to be important, so long as it is capable of expressing the nucleic acid in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the nucleic acid coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter.

In the examples given, the human cytomegalovirus (CMV) immediate early gene promoter (Example VI), the SV40 early promoter and the Rous sarcoma long terminal repeat (Example I), were used to obtain high-level expression of C-CAM. However, the use of other viral or mammalian cellular promoters which are well-
known in the art also is suitable to achieve expression of a CAM, provided that the levels of expression are sufficient for a given purpose.

By employing a promoter with well-known properties, the level and pattern of expression of a CAM following transfection can be optimized. For example, selection of a promoter which is active specifically in prostate cells, such as the prostate-specific antigen (PA) promoter (Watt et al., 1986), will permit tissue-specific expression of a CAM. Further, selection of a promoter that is regulated in response to specific physiologic signals can permit inducible expression of CAM. For example, with the nucleic acid encoding CAM being expressed from the human PAI-1 promoter, expression is inducible by tumor necrosis factor. Tables 1 and 2 list several elements/promoters which may be employed, in the context of the present invention, to regulate the expression of CAM. This list is not intended to be exhaustive of all the possible elements involved in the promotion of CAM expression but, merely, to be exemplary thereof.

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Aside from this operational distinction, enhancers and promoters are very similar entities.
Promoters and enhancers have the same general function of activating transcription in the cell. They are often overlapping and contiguous, often seeming to have a very similar modular organization. Taken together, these considerations suggest that enhancers and promoters are homologous entities and that the transcriptional activator proteins bound to these sequences may interact with the cellular transcriptional machinery in fundamentally the same way.

Below is a list of viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a CAM in an expression construct (Table 3A and Table 3B). Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a CAM.

**Table 3A**

<table>
<thead>
<tr>
<th>ENHANCER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoglobulin Heavy Chain</td>
</tr>
<tr>
<td>Immunoglobulin Light Chain</td>
</tr>
<tr>
<td>T-Cell Receptor</td>
</tr>
<tr>
<td>HLA DQ α and DQ β</td>
</tr>
<tr>
<td>β-Interferon</td>
</tr>
<tr>
<td>Interleukin-2</td>
</tr>
<tr>
<td>Interleukin-2 Receptor</td>
</tr>
<tr>
<td>MHC Class II 5</td>
</tr>
<tr>
<td>MHC Class II HLA-DRα</td>
</tr>
<tr>
<td>β-Actin</td>
</tr>
<tr>
<td>Muscle Creatine Kinase</td>
</tr>
<tr>
<td>Prealbumin (Transthyretin)</td>
</tr>
<tr>
<td>Elastase I</td>
</tr>
<tr>
<td>Metallothionein</td>
</tr>
<tr>
<td>ENHANCER</td>
</tr>
<tr>
<td>----------</td>
</tr>
<tr>
<td>Collagenase</td>
</tr>
<tr>
<td>Albumin Gene</td>
</tr>
<tr>
<td>α-Fetoprotein</td>
</tr>
<tr>
<td>τ-Globin</td>
</tr>
<tr>
<td>β-Globin</td>
</tr>
<tr>
<td>c-fos</td>
</tr>
<tr>
<td>c-HA-ras</td>
</tr>
<tr>
<td>Insulin</td>
</tr>
<tr>
<td>Neural Cell Adhesion Molecule (NCAM)</td>
</tr>
<tr>
<td>α1-Antitrypsin</td>
</tr>
<tr>
<td>H2B (TH2B) Histone</td>
</tr>
<tr>
<td>Mouse or Type I Collagen</td>
</tr>
<tr>
<td>Glucose-Regulated Proteins (GRP94 and GRP78)</td>
</tr>
<tr>
<td>Rat Growth Hormone</td>
</tr>
<tr>
<td>Human Serum Amyloid A (SAA)</td>
</tr>
<tr>
<td>Troponin I (TN I)</td>
</tr>
<tr>
<td>Platelet-Derived Growth Factor</td>
</tr>
<tr>
<td>Duchenne Muscular Dystrophy</td>
</tr>
<tr>
<td>SV40</td>
</tr>
<tr>
<td>Polyoma</td>
</tr>
<tr>
<td>Retroviruses</td>
</tr>
<tr>
<td>Papilloma Virus</td>
</tr>
<tr>
<td>Hepatitis B Virus</td>
</tr>
<tr>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>Gibbon Ape Leukemia Virus</td>
</tr>
</tbody>
</table>
Table 3B

<table>
<thead>
<tr>
<th>Element</th>
<th>Inducer</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT II</td>
<td>Phorbol Ester (TFA)</td>
</tr>
<tr>
<td></td>
<td>Heavy metals</td>
</tr>
<tr>
<td>MMTV (mouse mammary tumor</td>
<td>Glucocorticoids</td>
</tr>
<tr>
<td>virus)</td>
<td></td>
</tr>
<tr>
<td>β-Interferon</td>
<td>poly(rI)X</td>
</tr>
<tr>
<td></td>
<td>poly(rc)</td>
</tr>
<tr>
<td>Adenovirus 5 E2</td>
<td>Ela</td>
</tr>
<tr>
<td>Collagenase</td>
<td>Phorbol Ester (TPA)</td>
</tr>
<tr>
<td>Stromelysin</td>
<td>Phorbol Ester (TPA)</td>
</tr>
<tr>
<td>SV40</td>
<td>Phorbol Ester (TFA)</td>
</tr>
<tr>
<td>Murine MX Gene</td>
<td>Interferon, Newcastle Disease Virus</td>
</tr>
<tr>
<td>GRP78 Gene</td>
<td>A23187</td>
</tr>
<tr>
<td>α-2-Macroglobulin</td>
<td>IL-6</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Serum</td>
</tr>
<tr>
<td>MHC Class I Gene H-2kb</td>
<td>Interferon</td>
</tr>
<tr>
<td>HSP70</td>
<td>Ela, SV40 Large T Antigen</td>
</tr>
<tr>
<td>Proliferin</td>
<td>Phorbol Ester-TPA</td>
</tr>
<tr>
<td>Tumor Necrosis Factor</td>
<td>FMA</td>
</tr>
<tr>
<td>Thyroid Stimulating Hormone α</td>
<td>Thyroid Hormone</td>
</tr>
<tr>
<td>Gene</td>
<td></td>
</tr>
</tbody>
</table>

5. Selectable Marker

In certain embodiments of the invention, the delivery and expression of a nucleic acid encoding a CAM in a cell may be identified *in vitro* or *in vivo* by including a selectable marker in the expression construct and under the same transcriptional control as the nucleic acid encoding a CAM. The selectable marker would preferably involve a phenotypic change to the transfected cell to easily identify
expression. Usually the inclusion of a drug selection marker aids in the discovery and selection of transformants. Alternatively enzymes such as Herpes Simplex thymidine kinase (tk) or chloramphenicol acetyltransferase (CAT) may be employed. The selectable marker employed is not believed to be important, so long as it is capable of being expressed simultaneously to the nucleic acid encoding a CAM. Further examples of selectable markers are well known to one of skill in the art.

C. Polyadenylation Signal

Where a cDNA insert is employed, one will typically desire to include a polyadenylation signal to effect proper polyadenylation of the CAM transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. The inventors have employed the SV40 polyadenylation signal in that it was convenient and known to function well in the target cells employed.

D. Viral Expression Vectors

In preferred embodiments of the invention, the expression construct comprises a virus or engineered construct derived from a viral genome. The ability of certain viruses to enter cells via receptor-mediated endocytosis and to integrate into host cell genome and express viral genes stably and efficiently have made them attractive candidates for the transfer of foreign genes into mammalian cells (Ridgeway, 1988; Nicolas and Rubenstein, 1988; Baichwal and Sugden, 1986; Temin, 1986). The first viruses used as gene vectors were transforming DNA viruses including the papovaviruses (simian virus 40, bovine papilloma virus, and polyoma) (Ridgeway, 1988; Baichwal and Sugden, 1986) and adenoviruses (Ridgeway, 1988; Baichwal and Sugden, 1986). These have a relatively low capacity for foreign DNA sequences and have a restricted host spectrum. Furthermore, their oncogenic potential and cytopathic effects in permissive cells raise safety concerns. Murine retroviruses represent a more attractive alternative to the transforming DNA viruses (Nicolas and
Rubenstein, 1988; Temin, 1986). They can accommodate up to 8 kilobases of foreign genetic material and can be readily introduced in a variety of cell lines and laboratory animals (Nicolas and Rubenstein, 1988; Temin, 1986).

(i) Retroviruses

The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendents. The retroviral genome contains three genes, gag, pol, and env that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene, termed Ψ, functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a CAM is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol, and env genes but without the LTR and Ψ components is constructed (Mann et al., 1983). When a recombinant plasmid containing a human cDNA, together with the retroviral LTR and Ψ sequences is introduced into this cell line (by calcium phosphate precipitation for example), the Ψ sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann et al., 1983). The media containing the recombinant retroviruses is then collected, concentrated, and used for gene transfer. Retroviral vectors are able to
infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind et al., 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of lactose residues to the viral envelope. This modification could permit the specific infection of hepatocytes via sialoglycoprotein receptors.

A different approach to targeting of recombinant retroviruses was designed by Roux et al. in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux et al., 1989). Using antibodies against major histocompatibility complex class I and class I antigens, they demonstrated the infection of a variety of human cells that bore those surface antigens with an ecotropic virus in vitro (Roux et al., 1989).

There are certain limitations to the use of retrovirus vectors in all aspects of the present invention. For example, retrovirus vectors usually integrate into random sites in the cell genome. This can lead to insertional mutagenesis through the interruption of host genes or through the insertion of viral regulatory sequences that can interfere with the function of flanking genes (Varmus et al., 1981). Another concern with the use of defective retrovirus vectors is the potential appearance of wild-type replication-competent virus in the packaging cells. This can result from recombination events in which the intact Ψ' sequence from the recombinant virus inserts upstream from the gag, pol, env sequence integrated in the host cell genome. However, new packaging cell lines are now available that should greatly decrease the likelihood of recombination (Markowitz et al., 1988; Hersdorffer et al., 1990).

One limitation to the use of retrovirus vectors in vivo is the inability to produce retroviral vector titers greater than 10^6 infectious U/mL. Titers 10- to 1,000-fold higher are necessary for many in vivo applications.

Several properties of the retrovirus have limited its use in prostate cancer treatment (Stratford-Perricaudet and Perricaudet, 1991): (i) Infection by retrovirus
depends on host cell division. In human prostate cancer, very few mitotic cells can be
found in tumor lesions (Warner and Heston, 1991). (ii) The integration of retrovirus
into the host genome may cause adverse effects on target cells, because malignant
cells are high in genetic instability. (iii) Retrovirus infection is often limited by a
certain host range. (iv) Retrovirus has been associated with many malignancies in
both mammals and vertebrates. (v) The titer of retrovirus, in general, is 100- to 1,000-
fold lower than that of adenovirus.

(ii) Adenovirus

Knowledge of the genetic organization of adenovirus, a 36 kb, linear and
double-stranded DNA virus, allows substitution of a large piece of adenoviral DNA
with foreign sequences up to 7 kb (Grunhaus and Horwitz, 1992). In contrast to
retrovirus, the infection of adenoviral DNA into host cells does not result in
chromosomal integration because adenoviral DNA can replicate in an episomal
manner without potential genotoxicity. Also, adenoviruses are structurally stable, and
no genome rearrangement has been detected after extensive amplification.
Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage.
So far, adenoviral infection appears to be linked only to mild disease such as acute
respiratory disease in the human.

Adenovirus is particularly suitable for use as a gene transfer vector because of
its mid-sized genome, ease of manipulation, high titer, wide target-cell range, and
high infectivity. Both ends of the viral genome contain 100-200 base pair (bp)
inverted terminal repeats (ITR), which are cis elements necessary for viral DNA
replication and packaging. The early (E) and late (L) regions of the genome that
contain different transcription units are divided by the onset of viral DNA replication.
The E1 region (E1A and E1B) encodes proteins responsible for the regulation of
transcription of the viral genome and a few cellular genes. The expression of the E2
region (E2A and E2B) results in the synthesis of the proteins for viral DNA
replication. These proteins are involved in DNA replication, late gene expression, and
host cell shut off (Renan, 1990). The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at 16.8 m.u.) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promoter possess a 5' tripartite leader (TL) sequence which makes them preferred mRNAs for translation.

In the current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a single clone of virus from an individual plaque and examine its genomic structure.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by AD5 DNA fragments and constitutively expresses E1 proteins (Graham, et al., 1977). Since the E3 region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately 105% of the wild-type genome (Ghosh-Choudhury, et al., 1987), providing capacity for about 2 extra kb of DNA. Combined with the approximately 5.5 kb of DNA that is replaceable in the E1 and E3 regions, the maximum capacity of the current adenovirus vector is under 7.5 kb, or about 15% of the total length of the vector. More than 80% of the adenovirus viral genome remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1 deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available adenovirus vectors at high multiplicities of infection (Mulligan, 1993).
Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Other than the requirement that the adenovirus vector be replication defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup C is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the method of the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus E1 region. Thus, it will be most convenient to introduce the nucleic acid encoding CAM at the position from which the E1 coding sequences have been removed. However, the position of insertion of the CAM coding region within the adenovirus sequences is not critical to the present invention. The nucleic acid encoding CAM transcription unit may also be inserted in lieu of the deleted E3 region in E3 replacement vectors as described previously by Karlsson et al. (1986).

Adenovirus is easy to grow and manipulate and exhibits broad host range in vitro and in vivo. This group of viruses can be obtained in high titers, e.g., $10^9$-$10^{11}$ plaque-forming unit (PFU)/ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are expressed episomally, and therefore, have low genotoxicity to host cells. No side effects have been reported in studies of vaccination
with wild-type adenovirus (Couch et al., 1963; Top et al., 1971), demonstrating their safety and therapeutic potential as in vivo gene transfer vectors.

Adenovirus vectors have been used in eukaryotic gene expression (Levrero et al., 1991; Gomez-Foix et al., 1992) and vaccine development (Grunhaus and Horwitz, 1992; Graham and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Perricaudet et al., 1990; Rich et al., 1993). Experiments in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld et al., 1991; Rosenfeld et al., 1992), muscle injection (Ragot et al., 1993), peripheral intravenous injection (Herz and Gerard, 1993), and stereotactic inoculation into the brain (Le Gal La Salle et al., 1993).

(iii) Other Viral Vectors as Expression Constructs

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar et al., 1988) adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. They offer several attractive features for various mammalian cells (Friedmann, 1989; Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar et al., 1988; Horwich et al., 1990).

With the recent recognition of defective hepatitis B viruses, new insight was gained into the structure-function relationship of different viral sequences. In vitro studies showed that the virus could retain the ability for helper-dependent packaging and reverse transcription despite the deletion of up to 80% of its genome (Horwich et al., 1990). This suggested that large portions of the genome could be replaced with foreign genetic material. The hepatotropism and persistence (integration) were particularly attractive properties for liver-directed gene transfer. Chang et al. recently introduced the chloramphenicol acetyltransferase (CAT) gene into duck hepatitis B virus genome in the place of the polymerase, surface, and pre-surface coding
sequences. It was cotransfected with wild-type virus into an avian hepatoma cell line. Culture media containing high titers of the recombinant virus were used to infect primary duckling hepatocytes. Stable CAT gene expression was detected for at least 24 days after transfection (Chang et al., 1991).

5

METHODS FOR GENE TRANSFER

In order to effect expression of sense or antisense CAM constructs, the expression construct must be delivered into a cell. This delivery may be accomplished in vitro, as in laboratory procedures for transforming cell lines, or in vivo or ex vivo (see below), as in the treatment of certain disease states. As described above, the preferred mechanism for delivery is via viral infection where the expression construct is encapsidated in an infectious viral particle.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe et al., 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa et al., 1986; Potter et al., 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley et al., 1979), cell sonication (Fechheimer et al., 1987), gene bombardment using high velocity microprojectiles (Yang et al., 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for in vivo or ex vivo use.

Once the expression construct has been delivered into the cell the nucleic acid encoding CAM may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding CAM may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the
nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In one embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer in vitro, however, it may be applied for in vivo use as well. Dubensky et al. (1984) successfully injected polyomavirus DNA in the form of CaPO₄ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of CaPO₄ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a CAM may also be transferred in a similar manner in vivo and express CAM.

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein et al., 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which in turn provides the motive force (Yang et al., 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

Selected organs including the liver, skin, and muscle tissue of rats and mice have been bombarded in vivo (Yang et al., 1990; Zelenin et al., 1991). This may require surgical exposure of the tissue or cells, to eliminate any intervening tissue between the gun and the target organ, i.e., ex vivo treatment. Again DNA encoding a
CAM may too be delivered via this method and still be incorporated by the present invention.

In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991).


In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda et al., 1989). In other embodiments, the liposome may be complexed or employed in conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato et al., 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid in vitro and in vivo, then they are applicable for the present invention.

Other expression constructs which can be employed to deliver a nucleic acid encoding a CAM into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific
distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner et al., 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol et al., 1993; Perales et al., 1994) and epidermal growth factor (EGF) has also been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau et al. (1987) employed lactosyl-ceramide, a galactose-terminal asialganglioside, incorporated into liposomes and observed an increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a CAM also may be specifically delivered into a cell type such as prostate, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, the human prostate-specific antigen (Watt et al., 1986) may be used as the receptor for mediated delivery of a nucleic acid encoding a C-CAM in prostate cells.

In certain embodiments gene transfer may more easily be performed under ex vivo conditions. Ex vivo gene therapy refers to the isolation of cells from an animal, the delivery of a nucleic acid into the cells, in vitro, and then the return of the modified cells back into an animal. This may involve the surgical removal of tissue/organs from an animal or the primary culture of cells and tissues. Anderson et al. (US 5,399,346), and incorporated herein in its entirety, disclose of ex vivo therapeutic methods.

Primary mammalian cell cultures may be prepared in various ways. A preferred method is described, herein (Example I), for a human prostate carcinoma cell line (PC-3). However, many other cell cultures may be established from
tissues/cells in a similar manner, exemplary examples include prostate, breast, bladder, liver and intestines. In order for the cells to be kept viable while in vitro and contacting the cells with the expression construct it is necessary to ensure that the cells maintain contact with the correct ratio of oxygen and carbon dioxide and nutrients but are protected from microbial contamination. Cell culture techniques are well documented and are disclosed herein by reference (Freshner, 1992).

During in vitro culture conditions the expression construct may then deliver and express a nucleic acid encoding a C-CAM into the cells. Finally the cells may be reintroduced into the original animal, or administered into a distinct animal, in a pharmaceutically acceptable form by any of the means described below. Thus providing an ex vivo method of treating a mammal with a pathologic condition.

**C-CAM EXPRESSION CONSTRUCTS IN COMBINATION WITH OTHER THERAPIES**

Tumor cell resistance to DNA damaging agents represents a major problem in clinical oncology. One goal of current cancer research is to find ways to improve the efficacy of chemo- and radiotherapy by combining it with gene therapy. For example, the herpes simplex-thymidine kinase (HS-tK) gene, when delivered to brain tumors by a retroviral vector system, successfully induced susceptibility to the antiviral agent ganciclovir (Culver, et al., 1992). In the context of the present invention, it is contemplated that C-CAM replacement therapy could be used similarly in conjunction with chemo- or radiotherapeutic intervention.

To kill cells, such as malignant or metastatic cells, using the methods and compositions of the present invention, one would generally contact a "target" cell with a C-CAM expression construct and at least one DNA damaging agent. These compositions would be provided in a combined amount effective to kill or inhibit proliferation of the cell. This process may involve contacting the cells with the C-CAM expression construct and the DNA damaging agent(s) or factor(s) at the same time. This may be achieved by contacting the cell with a single composition or
pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the C-CAM expression construct and the other includes the DNA damaging agent.

Alternatively, the C-CAM treatment may precede or follow the DNA damaging agent treatment by intervals ranging from minutes to weeks. In embodiments where the DNA damaging factor and C-CAM expression construct are applied separately to the cell, one would generally ensure that a significant period of time did not expire between the time of each delivery, such that the DNA damaging agent and C-CAM expression construct would still be able to exert an advantageously combined effect on the cell. In such instances, it is contemplated that one would contact the cell with both agents within about 12-24 hours of each other and, more preferably, within about 6-12 hours of each other, with a delay time of only about 12 hours being most preferred. In some situations, it may be desirable to extend the time period for treatment significantly, however, where several days (2, 3, 4, 5, 6 or 7) to several weeks (1, 2, 3, 4, 5, 6, 7 or 8) lapse between the respective administrations.

It also is conceivable that more than one administration of either C-CAM or the DNA damaging agent will be desired. Various combinations may be employed, where C-CAM is "A" and the DNA damaging agent is "B":

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To achieve cell killing, both agents are delivered to a cell in a combined amount effective to kill the cell.

DNA damaging agents or factors are defined herein as any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage such
as, γ-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as "chemotherapeutic agents", function to induce DNA damage, all of which are intended to be of use in the combined treatment methods disclosed herein. Chemotherapeutic agents contemplated to be of use, include, e.g., adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin C, cisplatin (CDDP) and even hydrogen peroxide. The invention also encompasses the use of a combination of one or more DNA damaging agents, whether radiation-based or actual compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide. In certain embodiments, the use of cisplatin in combination with a C-CAM expression construct is particularly preferred as this compound.

In treating cancer according to the invention, one would contact the tumor cells with a DNA damaging agent in addition to the C-CAM expression construct. This may be achieved by irradiating the localized tumor site with DNA damaging radiation such as X-rays, UV-light, γ-rays or even microwaves. Alternatively, the tumor cells may be contacted with the DNA damaging agent by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a DNA damaging compound such as, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C, or more preferably, cisplatin.

The DNA damaging agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with a C-CAM expression construct, as described above.

Agents that directly cross-link nucleic acids, specifically DNA, are envisaged and are shown herein, to eventuate DNA damage leading to a synergistic antineoplastic combination. Agents such as cisplatin, and other DNA alkylating may be used. Cisplatin has been widely used to treat cancer, with efficacious doses used in clinical applications of 20 mg/m² for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intraumorally or intraperitoneally.
Agents that damage DNA also include compounds that interfere with DNA replication, mitosis, and chromosomal segregation. Such chemotherapeutic compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in clinical setting for the treatment of neoplasms, these compounds are administered through bolus injections intravenously at doses ranging from 25-75 mg/m² at 21 day intervals for adriamycin, to 35-50 mg/m² for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of nucleic acid precursors, and subunits also lead to DNA damage. As such a number of nucleic acid precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5-fluorouracil (5-FU), are preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Although quite toxic, 5-FU, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to 15 mg/kg/day being commonly used.

Other factors that cause DNA damage and have been used extensively include what are commonly known as γ-rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

The skilled artisan is directed to "Remington's Pharmaceutical Sciences" 15th Edition, chapter 33, in particular pages 624-652. Some variation in dosage will necessarily occur depending on the condition of the subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for
the individual subject. Moreover, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biologics standards.

The inventors propose that the regional delivery of C-CAM expression constructs to patients with C-CAM-linked cancers will be a very efficient method for delivering a therapeutically effective gene to counteract the clinical disease. Similarly, the chemo- or radiotherapy may be directed to a particular, affected region of the subjects body. Alternatively, systemic delivery of C-CAM expression construct or the DNA damaging agent may be appropriate in certain circumstances, for example, where extensive metastasis has occurred.

**NUCLEIC ACID ENCODING C-CAM OR C-CAM AS MARKERS**

In certain embodiments, a nucleic acid encoding a C-CAM or C-CAM may be employed for diagnostic purposes. The absence or reduced/increased level of C-CAM nucleic acid encoding a C-CAM may be indicative of a disease state such as cancer. Thereby using nucleic acid encoding a C-CAM or C-CAM as a marker. There are numerous methods, well known to one of skill in the art, that may be employed in detecting a nucleic acid encoding C-CAM or a C-CAM. Example I, describes two methods for detecting nucleic acids encoding a C-CAM; Southern and Northern analysis. The level of C-CAM message was used as a marker to indicate tumorigenicity.

An alternative approach would be to detect C-CAMs with immunoassays using antibodies that bind to C-CAM. Western Blotting and FACS analysis are also described in Example I. Both techniques were employed to detect C-CAM expression on the cell surface. It will be readily appreciated that detection is not limited to the above techniques, and that there are numerous other methods which may be encompassed by the present invention.
Other, preferred immunoassays are the various types of enzyme linked immunosorbent assays (ELISAs) and radioimmunoassays (RIA) known in the art. Immunohistochemical detection using tissue sections is also particularly useful.

In ELISAs, an anti-C-CAM antibody (such as Ab669, as disclosed herein) is immobilized onto a selected surface exhibiting protein affinity, such as a well in a polystyrene microtiter plate. Then, a test composition containing the cells or cellular material, such as a clinical sample, is added to the wells. After binding and washing to remove non-specifically bound immunocomplexes, the bound C-CAM may be detected. Detection is generally achieved by the addition of another anti-C-CAM antibody that is linked to a detectable label. This type of ELISA is a simple "sandwich ELISA". Detection may also be achieved by the addition of a second anti-C-CAM antibody, followed by the addition of a third antibody that has binding affinity for the second anti-C-CAM antibody, with the third antibody being linked to a detectable label.

In another exemplary ELISA, the samples containing the cellular material to be tested for the level of C-CAM, are immobilized onto the well surface and then contacted with the antiC-CAM antibodies. After binding and appropriate washing, the bound immunocomplexes are detected. Where the initial anti-C-CAM antibodies are linked to a detectable label, the immunocomplexes may be detected directly.

Again, the immunocomplexes may be detected using a second antibody that has binding affinity for the first anti-C-CAM antibody, with the second antibody being linked to a detectable label.

Competition ELISAs are also possible in which test samples compete for binding with known amounts of labeled C-CAM antigens or antibodies. The amount of reactive species in the unknown sample is determined by mixing the sample with the known labelled species before or during incubation with coated wells. The presence of reactive species in the sample acts to reduce the amount of labeled species available for binding to the well and thus reduces the ultimate signal.
Irrespective of the format employed, ELISAs have certain features in common, such as coating, incubating or binding, washing to remove non-specifically bound species, and detecting the bound immune complexes. These are described as follows:

In coating a plate with either antigen or antibody, one will generally incubate
the wells of the plate with a solution of the antigen or antibody, either overnight or for a specified period of hours. The wells of the plate will then be washed to remove incompletely adsorbed material. Any remaining available surfaces of the wells are then "coated" with a non-specific protein that is antigenically neutral with regard to the test antisera. These include bovine serum albumin (BSA), casein and solutions of milk powder. The coating allows for blocking of non-specific adsorption sites on the immobilizing surface and thus reduces the background caused by non-specific binding of antisera onto the surface.

In ELISAs, it is probably more customary to use a secondary or tertiary detection means rather than a direct procedure. Thus, after binding of the C-CAM or anti-C-CAM antibody to the well, coating with a non-reactive material to reduce background, and washing to remove unbound material, the immobilizing surface is contacted with the clinical or biological sample to be tested under conditions effective to allow immune complex (antigen/antibody) formation. Detection of the immune complex then requires a labeled secondary binding ligand or antibody, or a secondary binding ligand or antibody in conjunction with a labeled tertiary antibody or third binding ligand.

"Under conditions effective to allow immune complex (antigen/antibody) formation" means that the conditions preferably include diluting the antigens and antibodies with solutions such as BSA, bovine gamma globulin (BGG) and phosphate buffered saline (PBS)/Tween. These added agents also tend to assist in the reduction of non-specific background.

The suitable conditions also mean that the incubation is at a temperature and for a period of time sufficient to allow effective binding. Incubation steps are
typically from about 1 to 2 to 4 hours, at temperatures preferably on the order of 25°
to 27°C, or may be overnight at about 4°C or so.

Following all incubation steps in an ELISA, the contacted surface is washed so
as to remove non-complexed material. Washing often includes washing with a
solution of PBS/Tween, or borate buffer. Following the formation of specific
immune complexes between the test sample and the originally bound material, and
subsequent washing, the occurrence of even minute amounts of immune complexes
may be determined.

To provide a detecting means, the second or third antibody will have an
associated label to allow detection. Preferably, this will be an enzyme that will
generate color development upon incubating with an appropriate chromogenic
substrate. Thus, for example, one will desire to contact and incubate the first or
second immune complex with a urease, glucose oxidase, alkaline phosphatase or
hydrogen peroxidase-conjugated antibody for a period of time and under conditions
that favor the development of further immune complex formation (e.g., incubation for
2 hours at room temperature in a PBS-containing solution such as PBS-Tween).

After incubation with the labeled antibody, and subsequent to washing to
remove unbound material, the amount of label is quantified, e.g., by incubation with a
chromogenic substrate such as urea and bromocresol purple or 2,2'-azino-di-(3-ethyl-
benzthiazoline-6-sulfonic acid [ABTS] and H₂O₂, in the case of peroxidase as the
enzyme label. Quantification is then achieved by measuring the degree of color
generation, e.g., using a visible spectra spectrophotometer.

PHARMACEUTICAL COMPOSITIONS AND ROUTES OF
ADMINISTRATION

Where clinical application of an expression construct comprising a nucleic
acid encoding C-CAM is contemplated, it will be necessary to prepare the complex as
a pharmaceutical composition appropriate for the intended application. Generally this
will entail preparing a pharmaceutical composition that is essentially free of pyrogens,
as well as any other impurities that could be harmful to humans or animals. One also
will generally desire to employ appropriate salts and buffers to render the complex
stable and allow for complex uptake by target cells.

Aqueous compositions of the present invention comprise an effective amount
of the expression construct and nucleic acid, dissolved or dispersed in a
 pharmaceutically acceptable carrier or aqueous medium. Such compositions can also
be referred to as inocula. The phrases "pharmaceutically or pharmacologically
acceptable" refer to molecular entities and compositions that do not produce an
adverse, allergic or other untoward reaction when administered to an animal, or a
human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes
any and all solvents, dispersion media, coatings, antibacterial and antifungal agents,
isotonic and absorption delaying agents and the like. The use of such media and
agents for pharmaceutical active substances is well known in the art. Except insofar
as any conventional media or agent is incompatible with the active ingredient, its use
in the therapeutic compositions is contemplated. Supplementary active ingredients
also can be incorporated into the compositions.

Solutions of the active compounds as free base or pharmacologically
acceptable salts can be prepared in water suitably mixed with a surfactant, such as
hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid
polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of
storage and use, these preparations contain a preservative to prevent the growth of
microorganisms.

The expression constructs and delivery vehicles of the present invention may
include classic pharmaceutical preparations. Administration of therapeutic
compositions according to the present invention will be via any common route so long
as the target tissue is available via that route. This includes oral, nasal, buccal, rectal,
vaginal or topical. Alternatively, administration will be by orthotopic, intradermal
subcutaneous, intramuscular, intraperitoneal, or intravenous injection. Such
compositions would normally be administered as pharmaceutically acceptable
compositions that include physiologically acceptable carriers, buffers or other excipients.

The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers and the like may be used. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, etc. Intravenous vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to well known parameters.

Additional formulations which are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When the route is topical, the form may be a cream, ointment, salve or spray.

An effective amount of the therapeutic agent is determined based on the intended goal, for example (i) inhibition of tumor cell proliferation or (ii) elimination of tumor cells. The term "unit dose" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in
association with its administration, i.e., the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit dose, depends on the subject to be treated, the state of the subject and the protection desired. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual.

Kits

All the essential materials and reagents required for inhibiting tumor cell proliferation, transforming cells or detecting cancer cells, may be assembled together in a kit. This generally will comprise selected expression constructs. Also included may be various media for replication of the expression constructs and host cells for such replication. Such kits will comprise distinct containers for each individual reagent.

When the components of the kit are provided in one or more liquid solutions, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being particularly preferred. For in vivo use, the expression construct may be formulated into a pharmaceutically acceptable syringeable composition. In this case, the container means may itself be an inhalent, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, such as the prostate or breast, injected into an animal, or even applied to and mixed with the other components of the kit.

The components of the kit may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means.

The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, e.g., injection or blow-molded plastic containers into which the desired vials are retained.
Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/admistration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalent, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

* * * * * * * *

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE I: C-CAM1 - Transfected PC-3 Cells

A. Materials and Methods

1. Plasmid Construction and Transfection into PC-3 and NbE Cells

To construct sense and antisense C-CAM expression plasmids, a cDNA insert (1.7 kb) containing the entire coding sequence for C-CAM1 (Lin and Guidotti, 1989; Lin et al., 1991, SEQ ID NO:1) was cloned into the SalI site of the vector pRSN (Low et al., 1991), a mammalian expression vector carrying neomycin acetyltransferase gene (neo'), which confers neomycin resistance. The C-CAM1 cDNA was placed under the control of both the Rous sarcoma virus long terminal repeat and the SV40 early promoter. The orientation of C-CAM1 in pRSN was determined by restriction mapping. Digestion of sense clones with BamHI generates four fragments of; 2.7 Kb,
3.4 Kb, 2.1 Kb and 0.7 Kb in length. Digestion of antisense clones generates fragments of; 2.7 Kb, 3.4 Kb, 1.6 Kb and 1.2 Kb in length.

To produce transfected cells, 5 μg of C-CAM1 expression plasmid mixed in 10 μl of Transfectam (Promega, Madison, WI) was added to parental PC-3 or NbE cells grown in T medium (80% Dulbecco’s minimum essential medium plus 20% F-12 medium) containing 5% fetal bovine serum (FBS). To select for neomycin-resistant clones, the transfected cells were grown for 1 month in increasing concentrations of G418. The final concentration of G418 was 800 μg/ml and 1 mg/ml for PC-3 cells and NbE cells, respectively.

2. Cell Sorting by Flow Cytometry

Membrane fluorescence staining was performed on a single-cell suspension with an anti-C-CAM polyclonal antibody (Ab669; Culic et al., 1992) and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Cheung et al., 1993). FACS was performed with a dual-laser Vantage flow cytometer (Becton Dickinson, Mountain View, CA) delivering 50 mW at 488 nm with an Enterprise air-cooled laser. The flow cytometer was sterilized by a 30-min run with 70% ethanol and equilibration with sterile Isoton II (Coulter, Hialeah, FL) for 1 hr. Log green fluorescence was measured with a bandpass filter (530 nm/30 nm), and triggering mode was executed with forward light scatter set at a sufficiently high threshold to eliminate cell debris and dead cells (<5%). Drop delay was performed using the Autosort procedure (Becton Dickinson, Mountain View, CA). Analysis was performed using LYSYS II software (Becton Dickinson, Mountain View, CA).

3. Southern, Northern, and Western Blot Analyses

For Southern analysis, high-molecular-weight DNA was purified by the procedure of Davis et al. (1986). Twenty micrograms of high molecular weight DNA was digested with HindIII overnight at 37°C and then subjected to Southern blot analysis as described previously (Hsieh et al., 1993) with a C-CAM cDNA probe.
(Culic et al., 1992) or a neomycin acetyltransferase (neo') cDNA probe (Southern and Berg, 1982).

For Northern analysis, total cellular RNA was extracted from cells by using RNAzol B (Biotecx Laboratories Inc., Houston, TX) and a single-step purification protocol described by Chomczynski and Sacchi (1987). Twenty micrograms of RNA was subjected to Northern blot analysis by electrophoresis on a 0.9% agarose gel containing 2 M formaldehyde as described previously (Hsieh and Lin, 1994), and the blot hybridized with a random primer-labeled C-CAM cDNA probe (Culic et al., 1992), neo' cDNA probe or 28S RNA probe.

To isolate total cellular protein for Western analysis, the cells were trypsinized into a single-cell suspension and counted with a hemacytometer. Aliquots of the cell suspension from each clone were boiled in sodium dodecyl sulfate sample buffer, and Western blot analysis performed as described previously (Cheung et al., 1993) with anti-C-CAM antibody Ab669.

B. Results

1. C-CAM1-Transfected PC-3 Cells

To examine the role of C-CAM in prostate cancer progression, the C-CAM1 cDNA in the mammalian expression vector pRSN was transfected into a human prostate carcinoma cell line designated PC-3. This cell line is highly tumorigenic and found to be negative for C-CAM by both Northern and Western analyses. After transfection, the cells were grown in medium containing G418 to select for neomycin-resistant clones.

Immunofluorescence staining of the transfected PC-3 cells with anti-C-CAM antibody Ab669 was performed after 1 month of G418 selection. Although the immunofluorescence staining showed that C-CAM1 was expressed on the cell surface, only a small portion of cells (about 10%) reacted with the polyclonal antibody against C-CAM (FIG. 2A). This result suggested that only a small population of the transfected cells expressed C-CAM1. To increase the proportion of C-CAM1-positive
cells, the C-CAM1-positive cell population was enriched to 92% by fluorescence-activated cell sorting (FACS) (FIG. 2B). Two clones, PC-L1 and PC-L2, were isolated from the enriched population and characterized. Two additional clones, PC-L6 and PC-L9, were later isolated from this enriched population by using the same approach.

Southern blot analysis of genomic DNA isolated from various transfectants, digested with HindIII and hybridized with the neomycin acetyltransferase (neo') cDNA probe, revealed several different DNA integration patterns. As expected, the parental PC-3 cells were negative for neo'. PC-3 cells transfected with the PC-RSN vector alone (designated PC-RSN cells) showed a complex pattern of neo' integration. Two patterns of integration were seen among the four C-CAM1-positive clones, PC-L1 and PC-L2 having one type of integration and PC-L6 and PC-L9 another. These data indicate that these four C-CAM1-positive clones were derived from at least two different cells.

No C-CAM message was detected by Northern blot analysis in PC-3 or PC-RSN cells, suggesting that C-CAM was either not expressed or expressed in very low levels in these cells. In PC-L1 and PC-L2 cells, a single 3.0 kb C-CAM1 mRNA was detected. Two C-CAM transcripts were found in PC-L6 and PC-L9 cells, the 3.0 kb transcript and a slightly larger one. This larger transcript may represent a fusion of the C-CAM and neo' genes, because this band hybridized to both the C-CAM and Neo' probes. As predicted, the neo' mRNA was expressed in all transfected clones but not in the parental PC-3 cells. Hybridization with 28S rRNA, which served as an internal control, showed that similar amounts of RNA were loaded for each sample.

Western blot analysis with anti-C-CAM antibody Ab669 revealed similar profiles of C-CAM protein expression in all four C-CAM1 transfectants, while PC-3 and PC-RSN cells were negative for C-CAM expression. The C-CAM1 protein expressed from PC-3 transfectants was heterogeneous; the 105 kDa form was predominant. The heterogeneity may be due to different degrees of glycosylation since there are 16 potential N-linked glycosylation sites present in the extracellular
domain of C-CAM1. More C-CAM1 protein was detected in PC-L1 and PC-L2 cells
than in PC-L6 and PC-L9 cells, suggesting that C-CAM1 protein levels may be
regulated differently in these clones.

EXAMPLE II - Cell Adhesion and Growth Characteristics of Transfected PC-3
Cells

A. Materials and Methods

1. Cell Adhesion Assay

Cells were resuspended at a concentration of 1 x 10^6/ml. Cell suspensions (1
ml) in 1.5-ml Eppendorf tubes were mixed gently at room temperature to allow
formation of aggregates. Samples were taken over a 5-hr period, and the number of
single cells was determined with a hemacytometer. The aggregation of cells was
monitored as a decrease in the percentage of single cells.

2. In Vitro Growth Assay

The in vitro growth rate of C-CAM1-transfected PC-3 clones was measured
with three different assays. In one method, cells were grown in T-medium containing
5% FBS, trypsinized and counted with a hemacytometer 24, 48, and 96 hr after
plating. In a second method, 5,000 cells from each clone were grown on a 24-well
plate with T-medium containing 1% FBS. The medium was changed every 4 days.
The number of cells was determined every 48 hr using the crystal violet assay as
described in Gleave et al. (1991) and incorporated herein by reference. In a third
method, cells were plated as above and every 24 hours, fresh medium containing 0.1
mCi of [3H]-thymidine was added. [3H]-thymidine incorporation was determined
after a 2-hr incubation as described in Chang and Chung (1989) and incorporated
herein by reference.
B. Results

To test the intercellular adhesion of C-CAM1 expressed on the transfected PC-3 cells, the ability of single cells to form aggregates in suspension was measured. The time course of aggregate formation for PC-3, PC-RSN, PC-L1, PC-L2, PC-L6, and PC-L9 cells, as measured by the decrease in the number of single cells, is shown in FIG. 3. PC-3 and PC-RSN cells, which did not express the C-CAM1 protein, did not aggregate. In contrast, all of the C-CAM1 transfectants formed aggregates. These results indicate that the C-CAM1 expressed in PC-3 cells was able to elicit adhesion. Consistent with their higher level of C-CAM1 protein expression, PC-L1 and PC-L2 cells aggregated slightly more than PC-L6 and PC-L9 cells did.

The effect of C-CAM1 on cell growth also was analyzed. The four C-CAM1 transfected clones had growth rates (measured by counting the cells with a hemacytometer) of about 50% that of control cells. Similar results were obtained by measuring the increase in the number of cells by crystal violet staining (FIG. 4A) and by measuring the rate of DNA synthesis by [3H]thymidine incorporation (FIG. 4B). Crystal violet staining showed that PC-RSN cells had a growth rate similar to that of parental PC-3 cells. In contrast, the growth rates of the C-CAM1-transfected cells were about 25% that of the control cells 6 days after plating (FIG. 4A). By day 8, PC-L6 and PC-L9 cells were growing faster than PC-L1 and PC-L2 cells, perhaps because of the lower C-CAM1 levels in PC-L6 and PC-L9 cells. The rate of DNA synthesis, measured by [3H]thymidine incorporation, was noticeably lower than control levels in all of the C-CAM1-transfected cells (FIG. 4B). This finding suggests that the growth inhibition of C-CAM1-transfected cells may result from an alteration in the cell cycle.
EXAMPLE III- Suppression of Tumorigenicity of PC-3 Cells by C-CAM1 Expression

A. Materials and Methods

1. Measurement of Anchorage-Independent Growth

The soft-agar assay described by Giancotti and Ruoslahti (1990), and incorporated herein by reference, was used to measure the anchorage-independent growth of each transfected clone. Cells were plated onto 6-well plates, and colonies were scored in triplicate by two different investigators, 21 days after plating, by counting aggregates (>10 cells) in several randomly chosen areas.

2. Assessment of In Vivo Tumorigenicity

To determine the tumorigenicity of each transfected clone, 1 x 10⁶ cells/per site were injected at six sites in the flanks of 8- to 10-week-old male nude mice subcutaneously. Once a tumor became palpable, it was measured weekly and its size calculated using the formula for the volume of a hemiellipsoid, the geometric figure most nearly approximating the shape of the tumor: volume = length x width x height x 0.5236 (Rockwell et al., 1972).

B. Results

Normal cells must attach to a substratum to grow, whereas tumorigenic cells do not. As a result of this property, they can form colonies on soft-agar plates (Shin et al., 1975). To determine the effect of C-CAM1 expression on soft-agar colony formation by PC-3 cells, the inventors examined the anchorage-independent growth of the C-CAM1-transfected clones. These four clones formed significantly fewer colonies in soft-agar in three independent studies (Table 4A). In addition, the colonies of PC-L1, PC-L2, PC-L6, and PC-L9 cells were smaller than those of PC-3 and PC-RSN cells. These observations indicate that expression of C-CAM1 may affect the tumorigenicity of PC-3 cells.
Table 4A. Anchorage-Independent Growth of C-CAM1-Transfected PC-3 Cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>ND</td>
<td>100(^b)</td>
<td>100(^b)</td>
</tr>
<tr>
<td>PC-RSN</td>
<td>100(^b)</td>
<td>100±2</td>
<td>89±3</td>
</tr>
<tr>
<td>PC-L1</td>
<td>52±2</td>
<td>45±1</td>
<td>ND</td>
</tr>
<tr>
<td>PC-L2</td>
<td>36±4</td>
<td>45±1</td>
<td>30±1</td>
</tr>
<tr>
<td>PC-L6</td>
<td>ND</td>
<td>ND</td>
<td>50±3</td>
</tr>
<tr>
<td>PC-L9</td>
<td>ND</td>
<td>ND</td>
<td>60±2</td>
</tr>
</tbody>
</table>

\(^a\)Anchorage-independent growth was measured by triplicate counts of the colonies that had formed by day 21 after plating. The results are expressed as percentages of control (PC-RSN or PC-3) ± standard error. ND, not determined.

\(^b\)Control, defined as 100%.

Next, the ability of these cells to produce tumors in nude mice *in vivo* was measured. PC-3 cells previously have been shown to be highly tumorigenic when injected into nude mice (Kaighn *et al.*, 1979). To test the transfected cells, cells from each clone were injected into the flanks of male athymic nude mice subcutaneously and the incidence of tumor formation and the volumes of the tumors monitored. As shown in Table 4, 60 days after injection, the tumor incidence was 67-94% for PC-3 cells and PC-RSN cells. In contrast, a much lower tumor incidence was observed in mice injected with C-CAM1-transfected cells. In addition, parental PC-3- and PC-RSN-induced tumors generally arose within 3 to 4 weeks of injection, whereas injecting the same number of C-CAM1-transfected cells into nude mice produced fewer or no tumors within the first 30 days (Table 4B). In addition, the tumors produced from transfected cells were much smaller (FIG. 5A and FIG. 5B). These
data indicate that expression of C-CAM1 in PC-3 cells suppressed or delayed tumor growth in vivo.

Table 4B. Tumor Incidence Induced by C-CAM1-Transfected PC-3 Cells

<table>
<thead>
<tr>
<th>Clone</th>
<th>Test 1 Day 30</th>
<th>Test 1 Day 60</th>
<th>Test 2 Day 30</th>
<th>Test 2 Day 60</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>12/36(33%)</td>
<td>24/36(67%)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PC-RSN</td>
<td>29/36(80%)</td>
<td>34/36(94%)</td>
<td>12/18(67%)</td>
<td>17/18(94%)</td>
</tr>
<tr>
<td>PC-L1</td>
<td>2/36(5%)</td>
<td>12/36(33%)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PC-L2</td>
<td>0/36(0%)</td>
<td>2/36(5%)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PC-L6</td>
<td>ND</td>
<td>ND</td>
<td>0/18(0%)</td>
<td>0/18(0%)</td>
</tr>
<tr>
<td>PC-L9</td>
<td>ND</td>
<td>ND</td>
<td>0/18(0%)</td>
<td>4/18(22%)</td>
</tr>
</tbody>
</table>

*a*Tumors larger than 10 mm³ were considered to be positive. ND, not determined.

Taken together, these results demonstrated that expression of C-CAM1 markedly suppressed the tumorigenicity, as well as the tumor growth, of PC-3 cells.

EXAMPLE IV - Increased Tumorigenicity of NbE Cells by Transfection of Antisense C-CAM1 Plasmid

A. Materials and Methods

1. In Vitro Characterization of Mac Clones

Plasmid construction and transfection of NbE cells were as described in Example I. Individual Mac clones derived from NbE cells transfected with antisense C-CAM1 cDNA were characterized for cDNA integration and protein expression. For Southern blot analysis, 20 µg of high-molecular-weight DNA was isolated from each cell line, digested with HindIII at 37°C overnight, and then subjected to Southern blot analysis. The blot was hybridized with a neo¹ probe and a C-CAM
probe as described in Example 1. Total protein (5 μg) from each cell line was subjected to Western blot analysis with the anti-C-CAM antibody, Ab669. The relative levels of C-CAM protein was determined by densitometrical scanning.

After growing in 1 mg/ml G418 for 1 month, transfected cells (antisense-1, 1 x 10^6 cells/site) were injected into nude mice subcutaneously.

2. In Vitro Cell Cloning of NbE Sublines (Mac)

Tumors were excised from animals and placed in serum-free T-medium containing antibiotics. After removing excess skin and blood clots, tissues were rinsed with serum-free T-medium, 3 times. Tissues were cut into 3 mm^3 pieces and placed in a 6-well plate coated with FBS; then, a small amount of 10% FBS T-medium was added to cover each tumor for overnight incubation at 37°C. The medium was changed every 4 days and G418 (200 μg/ml) was added when epithelia started appearing on the dish. The concentration of G418 was gradually increased to a final concentration of 1 mg/ml within 1 month. The cells were then further cloned by the ring-cloning technique as described in Freshney (1987) and incorporated herein by reference.

B. Results

To further examine the tumor-suppressor activity of C-CAM1 in prostate cancer development, the inventors tested whether down-regulation of C-CAM in a non-tumorigenic cell line could lead to tumor formation in nude mice. The NbE cell line, a prostatic epithelial cell line derived from the ventral prostate of a Noble rat (Chung et al., 1989), was chosen because it expresses detectable levels of C-CAM and does not cause tumor formation when injected into nude mice or into the syngeneic host (Chung et al., 1989). The antisense C-CAM1 plasmid, constructed by inserting the full-length C-CAM1 cDNA into pRSN in the anti-sense orientation, was used to reduce C-CAM1 expression.
In the first study, NbE cells were transfected with an antisense C-CAM1 vector. Transfected cells were selected by increasing concentrations of G418. After growing in 1 mg/ml G418 for 1 month, transfected cells (antisense-1, 1 x 10^6 cells/site) were injected into nude mice subcutaneously. Sixty days later, tumors appeared at all the antisense-1 injection sites (Table 5A), but no tumors appeared where parental NbE cells had been injected. The antisense-induced NbE tumors were excised from the mouse and cells were cloned from these tumors. Since antisense-transfected NbE cells are resistant to G418 treatment and the cells derived from the nude mouse are not, the antisense-transfected NbE cells can be specifically selected from total tumor tissues by G418 selection. After G418 selection for one month, six sublines (named Mac-1 to Mac-6) were isolated from six individual tumors. The tumorigenicities of these six cell lines were tested together with several control NbE cell lines, as shown in Table 5B.

To confirm the previous finding, the same antisense vector was transfected into NbE cells to generate a second transfected NbE cells (antisense-2). Other control NbE cell lines, including those transfected with sense and vector controls, also were generated and selected under the conditions used in the first study. When these cell lines were injected into nude mice, the antisense vector induced tumors at an incidence of 50% appeared 40 days after injection (Table 5B), with an average tumor volume similar to that of the previous study (Table 5A), whereas no tumors developed in mice injected with sense and vector controls (Table 5B).

**Table 5A. Tumor Incidence and Tumor Growth Induced by Antisense C-CAM1-Transfected NbE Cells**

<table>
<thead>
<tr>
<th>NbE clones</th>
<th>Tumor incidence (%)</th>
<th>Tumor volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>parental</td>
<td>0/6 (0%)</td>
<td>0</td>
</tr>
<tr>
<td>antisense-1</td>
<td>6/6 (100%)</td>
<td>49±9</td>
</tr>
</tbody>
</table>
Table 5B. Tumor Incidence and Tumor Growth Induced by Antisense C-CAM1-Transfected NbE Cells

<table>
<thead>
<tr>
<th>NbE clones</th>
<th>Tumor incidence(^a)(%)</th>
<th>Tumor volume (mm(^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14 days</td>
<td>40 days</td>
</tr>
<tr>
<td>parental</td>
<td>0/18 (0%)</td>
<td>0/18 (0%)</td>
</tr>
<tr>
<td>plasmid control</td>
<td>0/18 (0%)</td>
<td>0/18 (0%)</td>
</tr>
<tr>
<td>sense</td>
<td>0/18 (0%)</td>
<td>0/18 (0%)</td>
</tr>
<tr>
<td>antisense-2</td>
<td>0/18 (0%)</td>
<td>9/18 (50%)</td>
</tr>
<tr>
<td>Mac-1</td>
<td>11/12(92%)</td>
<td>12/12(100%)</td>
</tr>
<tr>
<td>Mac-2</td>
<td>11/12(92%)</td>
<td>12/12(100%)</td>
</tr>
<tr>
<td>Mac-3</td>
<td>12/12(100%)</td>
<td>12/12(100%)</td>
</tr>
<tr>
<td>Mac-4</td>
<td>12/12(100%)</td>
<td>12/12(100%)</td>
</tr>
<tr>
<td>Mac-5</td>
<td>12/12(100%)</td>
<td>12/12(100%)</td>
</tr>
<tr>
<td>Mac-6</td>
<td>12/12(100%)</td>
<td>12/12(100%)</td>
</tr>
</tbody>
</table>

* Tumors larger than 10 mm\(^3\) were considered to be positive.

Histopathologic examination of hematoxylin- and eosin-stained tumor tissues from these antisense-2 tumors revealed that they were anaplastic and poorly differentiated. In the same in vivo tumorigenicity study, all six anti-sense sublines, Mac-1 to Mac-6, showed aggressive tumor formation with almost 100% tumor incidence, and palpable tumors 14 days after inoculation. These results indicate that transfection of antisense C-CAM1 into NbE cells changed the cells from non-tumorigenic to highly tumorigenic.

Southern analyses were used to determine the clonality of antisense NbE-derived sublines, Mac-1 to Mac-6. All Mac clones hybridized with both the neo\(^r\) and the C-CAM probes; however, two patterns of integration were observed. Mac-1, Mac-2, Mac-5, and Mac-6 had the same DNA integration pattern, while Mac-3 and
Mac-4 had a different pattern. These results indicate that these six sublines originated from two different clones.

Western immunoblotting indicated that the levels of C-CAM were significantly (10- to 100-fold) lower in all six sublines than in the parental cells.

Results from the densitometrical scanning, were as follows: parental (100%), plasmid control (85±7%), Mac-1 (9±1%), Mac-2 (3±1%), Mac-3 (4±1%), Mac-4 (1±0%), Mac-5 (1±0%), Mac-6 (6±1%). Thus, the increased tumorigenicity of the antisense C-CAM-transfected cells correlated with their reduced C-CAM levels.

EXAMPLE V - Expression of C-CAM During Human Prostate Development

A. Materials and Methods

1. Immunohistochemistry

Tissue samples from radical prostatectomy (25 patients) and transurethral resection of the prostate (1 patient) were obtained from the Department of Pathology at the M.D. Anderson Cancer Center. The tissue samples were immediately placed in tissue blocks with O.C.T. compound (Miles, Elkhart, IN) and frozen at -20°C. Both prostate carcinomas and normal prostatic tissues were included in this study. Serial 4 µm-thick sections were cut from each sample with a cryostat. One section was stained with hematoxylin and eosin for pathological evaluation and grading; adjacent sections were used for immunohistochemical staining. The prostate adenocarcinomas were graded according to the Gleason grading system. Prostatic intraepithelial neoplasia (PIN) was classified as low grade (PIN1) and high grade (PIN2 and PIN3) as agreed at the 1989 Workshop on Prostatic Dysplasia (Drago et al., 1989). In addition, prostate samples representative of prostate gland development, such as fetal prostates (30- and 36-week-old) and a prostate from a 13-year-old boy were included in the study. These samples were provided as frozen sections by Sherwood et al. (1991).
For immunoperoxidase staining, tissue was placed on silicone coated slides, which were then rinsed in phosphate-buffered saline (PBS). The tissue was fixed in a solution of 95% methanol and 5% acetic acid at -20°C for 15 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol at room temperature for 15 min. After the tissue was washed with PBS three times for 5 min. each, Superblock (Scytek Laboratories, Logan, UT) was applied for 20 min. at room temperature. Slides were then incubated with the polyclonal antibody specific against C-CAM (Ab 669, 1:2000 dilution in PBS containing 1% bovine serum albumin) overnight at 4°C. The negative controls were incubated with preimmune serum. A biotin-avidin amplification system using a StrAviGen™ Super Sensitive kit (Biogenex Laboratories, San Ramon, CA) with diaminobenzidine (DAB) as the chromogen was applied. A light green dye (Sigma Chemical Co., St. Louis, MO) was used for background counterstaining.

Immunohistochemical staining was evaluated by three observers. The pattern of staining was classified as positive with a continuous staining (staining in a continuous pattern surrounding any given gland), positive with a discontinuous staining (staining in a broken or discontinuous pattern surrounding any given gland), or negative staining (no staining at all).
Table 6: C-CAM Staining in the Normal Adult Prostate, BPH, PIN (A) and Primary Prostate Carcinomas (B)

(A)  
<table>
<thead>
<tr>
<th>Histology</th>
<th>Zonal Origin</th>
<th>Cases</th>
<th>Staining Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Continuous</td>
</tr>
<tr>
<td>Normal</td>
<td>Peripheral</td>
<td>20</td>
<td>20 (100%)</td>
</tr>
<tr>
<td>Normal</td>
<td>Central</td>
<td>2</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>Normal</td>
<td>Transition</td>
<td>7</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>Atrophy</td>
<td>Peripheral/ Transition</td>
<td>17</td>
<td>17 (100%)</td>
</tr>
<tr>
<td>BPH</td>
<td>Transition</td>
<td>5</td>
<td>2 (40%)</td>
</tr>
<tr>
<td>PIN^d</td>
<td>Peripheral</td>
<td>12</td>
<td>7 (58%)</td>
</tr>
</tbody>
</table>

(B)  

<table>
<thead>
<tr>
<th>Gleason Score</th>
<th>Zonal Origin</th>
<th>Cases</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-7</td>
<td>Transition</td>
<td>3</td>
<td>0</td>
<td>3 (100%)</td>
</tr>
<tr>
<td></td>
<td>Peripheral</td>
<td>16</td>
<td>0</td>
<td>16 (100%)</td>
</tr>
<tr>
<td>8-10</td>
<td>Peripheral</td>
<td>1</td>
<td>0</td>
<td>1 (100%)</td>
</tr>
<tr>
<td></td>
<td>Not determined</td>
<td>2</td>
<td>0</td>
<td>2 (100%)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>22</td>
<td>22</td>
<td>22 (100%)</td>
</tr>
</tbody>
</table>

^aContinuous: Uniform staining throughout the entire basal cell layer of each gland.
^bDiscontinuous: Non-uniform staining of the basal cell layer surrounding each gland.
^cIn all 3 of these cases the inventors observed a mixed staining pattern: some BPH glands had discontinuous staining, whereas other BPH glands had negative staining.
^dHigh grade PIN (PIN2 and PIN3).
^eIn 4 of 5 of these cases the inventors observed a mixed staining pattern: some PIN glands had discontinuous staining, whereas other PIN glands had continuous staining.
^fIn the fifth case of PIN there was solely discontinuous staining.
^gZone not able to be determined.
B. Results

Arising from the urogenital sinus and the vesicourethral components of the cloaca. The bud stage (20-30 weeks gestation) is characterized by solid epithelial buds at the ends of ducts without a recognizable lumen (Xia et al., 1990). Using cell type-specific cytokeratin antibodies for either basal or luminal cells, Sherwood et al. clearly showed that only basal cells were present in these epithelial buds (1991). By 36 weeks of gestation, some buds are still visible; however, the majority have developed into acinotubular structures with identifiable lumens (Xia et al., 1990).

To understand the expression pattern of C-CAM during prostate development, the inventors studied the same 30-week- and 36-week-old fetal prostate specimens by immunohistochemical staining using a polyclonal antibody specific against C-CAM, Ab 669. Recent data indicated that Ab 669 raises against rat C-CAM molecule can cross react with the human counterpart BGP-1 (Gao and Knowles, 1993) but appears not to cross-react with CEA and other immunoglobulin-like CAM's (Gao and Knowles, 1993). In the 30-week-old fetal prostate, C-CAM expression could be detected as typical plasma membrane staining associated with multiple basal cell layers in the epithelial buds. In contrast, the surrounding stromal compartment stained negatively.

In addition to epithelial buds in the 36-week-old fetal prostate, there were acino-tubular structures with identifiable lumens. C-CAM expression localized predominantly in the basal cell layers of these maturing glands. Luminal epithelium showed weak if any staining. Furthermore, tissue obtained from a 13-year-old prostate exhibited a continuous positive staining of C-CAM associated with the basal cell layer, and again the luminal epithelium showed weak if any staining. In contrast, the antibody controls showed a negative C-CAM staining. In the normal adult prostate gland, C-CAM expression showed the same positive staining pattern in the basal cell layer as was observed in the fetal prostates. Taken together, these data
indicate that C-CAM expression in the normal human prostate gland is associated with basal cells throughout the early fetal stage into adulthood.

In contrast, the rat ventral prostate shows a different staining pattern; steady-state levels of C-CAM are detected in the luminal epithelium from intact animals. However, elevated levels of C-CAM are detected in the basal cell layer only after androgen deprivation (Hsieh and Lin, 1994). This difference in C-CAM staining between the two species may be due to differences in the proliferative potential of luminal epithelium. For example, in the intact rat prostate, proliferative activity is found in the luminal epithelium (English et al., 1987). In the human prostate, however, the majority of proliferative activity is confined to the basal cell population (Bonkhoff et al., 1994).

Consistent with the results from fetal prostate development, the inventors observed a continuous plasma membrane staining of C-CAM predominantly associated with basal cells in the normal glandular epithelium from the peripheral, central and transition zones of the prostate. As summarized in Table 3A, the inventors observed the same continuous staining pattern of C-CAM in the basal cell layer in all specimens examined (29 cases) regardless of zonal origin. On the other hand, luminal cells showed weak if any staining. In addition, atrophic glands (17 cases) with a distinct basal cell layer exhibited a continuous C-CAM staining pattern in the basal cell layer in any given gland (Table 3A).

2. Decreased Expression of C-CAM in BPH and PIN

Benign prostatic hyperplasia (BPH), often found in the transition zone/periurethral gland region of the human prostate (McNeal, 1978), is considered a hyperplastic growth of epithelium and/or mesenchyme. In this study, an overall decrease in C-CAM staining was observed in the epithelial compartment from BPH glands compared with adjacent normal glands in the transition zone. The overall C-CAM staining pattern of basal epithelium from BPH glands can be divided into two categories. First, as shown in Table 3A, in 40% of specimens examined, the basal
epithelium from BPH glands showed a continuous staining pattern of C-CAM with a similar staining to normal glands, but the majority (60%) of specimens showed either a discontinuous staining pattern in the basal layer or no staining at all. However, in the adjacent normal (nonhyperplastic) glands from the same tissue section, the basal cell layers exhibited a continuous C-CAM staining. Therefore, decreased C-CAM expression may be associated with hyperplasia of the prostatic epithelium.

Prostatic intraepithelial neoplasia (PIN) is considered a precursor of prostate carcinoma (McNeal and Bostwick, 1986; Troncoso et al., 1989). Therefore, it is important to determine C-CAM expression in PIN. The inventors' finding on PIN are limited to high grade (PIN2 and PIN3) lesions that could be reproducibly identified on the frozen sections. Because low grade PIN (PIN1) could not be unequivocally distinguished from epithelial hyperplasia or other minor epithelial atypias on frozen sections, it was not included in the inventors' evaluation.

As summarized in Table 3A, the overall C-CAM staining pattern associated with basal cells is altered in high grade PIN glands. For example, although some PIN glands still exhibited a continuous C-CAM staining pattern in the basal cell layer, adjacent PIN glands had a discontinuous pattern of C-CAM staining along the basal cell layer. Very often, this discontinuous pattern of C-CAM staining associated with PIN was seen adjacent to areas of carcinoma, which were completely negative for C-CAM staining (FIG. 4A and FIG. 4B). Notably, the inventors also observed a mixed pattern of both continuous and discontinuous staining associated with the basal layer within a single PIN gland.

These observations suggested that the altered and decreased C-CAM expression may be associated with the continuous progression from normal prostatic epithelium to premalignant transformation and finally to carcinoma (Bostwick and Brawer, 1987). Moreover, these results indicated that altered C-CAM expression may be an early event associated with prostate cancer development.
3. **Absent Expression of C-CAM in Primary Prostate Carcinomas**

Prostate cancer is a multifocal disease with a wide range of histologic patterns and biologic potential. The heterogeneous staining pattern of C-CAM in premalignant PIN glands prompted the examination of C-CAM expression in prostate cancers of different histologic grade and zonal origin. In all, 22 prostate carcinoma specimens examined, a complete absence of C-CAM staining was observed regardless of tumor grade and zonal origin (FIG. 4 and Table 3B). The carcinomas studied were moderately and poorly differentiated tumors (Gleason score 5-10). Although the inventors did not examine well-differentiated carcinomas (Gleason score 2-4), areas of histologic grade 2 present focally among the moderately differentiated tumors also were negative.

In contrast, normal glands from the same sections still retained a continuous staining pattern of C-CAM, indicating that the absence of staining in carcinoma was not due to staining artifact (FIG. 4C and FIG. 4D). All pre-immune serum controls were negative. Thus, the expression of C-CAM inversely correlated with the appearance of carcinoma. The complete loss of C-CAM even in the foci within moderately-differentiated tumors suggests that the loss of C-CAM occurs early in malignant transformation.

Consistent with the absence of C-CAM expression in human prostate cancer specimens, C-CAM also was not detected in several established human prostate cancer cell lines, including PC-3 (Example 1), and DU-145 and LNCaP cells.

**EXAMPLE VI - Construction and Analysis of the C-CAM1 Recombinant Adenovirus**

A. **Materials and Methods**

1. **Construction and large-scale production of the C-CAM1 recombinant adenovirus**

To construct a recombinant adenovirus containing the C-CAM1 gene, as shown in FIG. 6, C-CAM1 cDNA (Lin and Guitti, 1989; Cheung *et al.*, 1993a; SEQ
ID NO:1) was cloned into the EcoRI site of the multiple cloning sites of the pBSK vector (Stratagene, La Jolla, CA). Either a sense or antisense strand of C-CAM1 cDNA was directionally cloned into the HindIII and NotI sites of a shuttle vector (pAdE1CMV/pA). So that its expression was under the control of a cytomegalovirus (CMV) promoter, one of the very strong promoters (Boshart et al., 1985). The recombinant adenovirus was generated from cotransfection of the shuttle vector (30 μg) with the C-CAM1 cDNA insert and a pJM17 vector (20 μg) carrying both adenoviral genome (Ad5) and pBR322 sequences.

The two vectors were cotransfected into 293 cells using DOTAP reagent (Boehringer-Mannheim, Indianapolis, IN) according to the manufacture's protocol. The pBR322 sequences in pJM17 make the entire viral DNA too large to be packed into protein coat, which prevents the appearance of wild type virus (Graham and Prevec, 1991). In this study, two replication-deficient recombinant viruses, one containing the sense orientation of C-CAM1 cDNA (AdCAM902) and the other containing the antisense orientation of C-CAM1 cDNA (AdCAM101), were generated from homologous recombination in a packaging cell line (293 cells). Each clone of viruses was isolated from a single plaque after reinfecting 293 cells (Graham and Prevec, 1991). Subsequently, a large amount of viral stocks were produced from 293 cells and purified by Inland Laboratories, Inc. (Austin, TX). The average titers of viral stocks were estimated to be 1 x 10^{11} virions/ml as determined by A_{260} assay; or 1 x 10^{11} plaque forming units (pfu/ml) for AdCAM101 and 1 x 10^{10} pfu/ml for AdCAM902 as determined by a plaque assay.

2. Analysis of the structure of the recombinant adenovirus by PCR

Adenoviral DNA was prepared as described previously (Zhang et al., 1993). PCR \textsuperscript{TM} (Polymerase chain reaction) was performed in a 50-ml reaction mixture containing 4 mM MgCl\textsubscript{2}, 50 mM KCl, 0.1% Triton\textsuperscript{X-100}, 200 mM of each deoxyribonucleotide triphosphate, 10 mM Tris-HCl (pH 9.0), 2 mM of the different primer sets (primer set A \textsuperscript{5'} GATCCAACACAAGGAAATTC\textsuperscript{3'},
5’-TCATTCTTTTTGACTAC-3’; primer set B [5’GGCCCACCCCTTGGCTTC3’, 5’TGTAACTTATAAAGCTGC3’]; or primer set C [5’TCTTTCTCAGCAGCTGGTG3’, 5’CATCTGAACTCAAGCGTG3’], and 1.0 unit of Taq DNA polymerase (Promega, Madison, WI). The reactions were carried out for 30 cycles, and each cycle was performed at 94°C for 0.5 min, at 56°C for 0.5 min, and 72°C for 1 min.

3. **Determination of viral infectivity of PC-3 cells by Fluorescent-activated cell scanning analysis**

   PC-3 cells (5 x 10^5 cells) were plated on a 65-mm plate for 24 h, then different concentrations of viruses were added and incubated at 37°C for 24 h. Cells were trypsinized into a single-cell suspension and incubated with either pre-immune serum or anti-C-CAM polyclonal antibody (Ab669) and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies as described by Cheung et al., 1993a. Percentage of positive cells was determined by a dual-laser Vantage flow cytometer (Becton Dickinson, Mountain View, CA) as described in Example I.

4. **Determination of C-CAM1 expression by C-CAM1 recombinant adenovirus using Northern and Western analyses**

   To determine the levels of C-CAM1 expression in viral-infected cells, the inventors performed both Northern and Western assays as described in Example I. For the dose-dependent studies, PC-3 cells (5x10^5 cells) were infected with either AdCAM101 or AdCAM902 at various ratios of virions:cells (0:1, 1:1, 5:1, and 20:1) for 24 h. Cells were harvested for the determination of C-CAM1 mRNA and protein levels. For the time course studies, the same amount of PC-3 cells was infected with either AdCAM101 or AdCAM902 at a 5:1 virion:cell ratio and medium was changed every 3 days. For the Northern blot analysis, cells were harvested for the determination of C-CAM1 mRNA levels at day 4, 7, 10 and 14. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. For the Western
blot analysis, cells were harvested for the determination of C-CAM1 protein levels at
day 6, 14 an 20.

B. Results

To ensure the completion of homologous recombination and the presence of
C-CAM in these recombinant adenoviruses, the inventors performed PCR™ using
different sets of primers as described in Materials and Methods. As shown in FIG. 7,
using a C-CAM-specific primer set (i.e., primer set A) from the C-terminal portion
(from amino acid 413 to amino acid 519) of C-CAM (Lin and Guidotti, 1989), the
inventors were able to amplify a 0.3 kB transcript from both AdCAM101 and
AdCAM902 DNA, indicating that both adenoviruses contained the C-CAM
sequences.

Also, using primer set B for the flanking sequences from the human CMV
promoter and SV40 polyadenylation site, a 2.1 kB full-length C-CAM cDNA
transcript was detected in both viruses. These data indicated that the C-CAM insert
was located between CMV promoter and SV40 polyadenylation. Moreover, using an
adenovirus-specific primer (i.e., primer set C), an expected 0.86 kB PCR™ transcript
appeared from both viruses, indicating that both viruses were generated from
homologous recombination. Taken together, these data indicate that these two viruses
contain the expected gene structure.

To characterize the infectivity of adenovirus to prostatic cancer cells, PC-3
cells (5x10⁵ cells) were plated in a 65-mm dish overnight prior to infection, then
various ratios of viral particles per cell were added to cells and incubated at 37°C for
1, 2, and 4 day. The efficiency of viral infection was determined by fluorescent-
activated cell scanning based on positive membrane staining of C-CAM1. As shown
in FIG. 8, the background fluorescence was set at 20 FITC units based on the
scanning of PC-3 cells infected with buffer control (FIG. 8A, FIG. 8E, FIG. 8I). PC-3
cells infected with AdCAM101 (FIG. 8B, FIG. 8F, FIG. 8J) gave the same
fluorescence profile as for the buffer control indicating that cells infected with
AdCAM101 did not express C-CAM protein. On the other hand, C-CAM was expressed by PC-3 cells infected with AdCAM902 (FIG. 8C, FIG. 8D, FIG. 8G, FIG. 8H, FIG. 8K, FIG. 8L).

When the inventors determined the percentage of positive cells after AdCAM902 infection for one day, the inventors found that 48% of cells expressed C-CAM1 when the cells were infected at a 100:1 virion:cell ratio (FIG. 8C), and 93% of cells expressed C-CAM when infected at a 1000:1 ratio (FIG. 8D), which is comparable to other report using β-gal adenovirus (Li et al., 1993). These data indicate that C-CAM1 expression in AdCAM902-infected PC-3 cells is dose-dependent. On the other hand, the inventors observed that maximal infection (~93%) plateaued within 24 h at the saturated viral concentration (FIG. 8D, FIG. 8H, FIG. 8L). However, under the subsaturated viral concentration, the infectivity rate increased from 48% at day 1 to 77% at day 4 in a time-dependent manner (FIG. 8C, FIG. 8G, FIG. 8K), suggesting that this recombinant adenovirus can maintain its infectivity at least 4 days.

When PC-3 cells were infected with sense recombinant adenovirus (AdCAM902), both Northern and Western blot analyses showed that C-CAM1 expression could be detected 24 h after infection and that the level of C-CAM1 expression depended on the number of viruses used for infection. Detection of C-CAM expression was easily observed at ratios of 5:1 and 20:1, virions:cell. A much weaker signal was detected from PC-3 cells infected with AdCAM101, using a C-CAM1 cDNA probe, suggesting the presence of the antisense C-CAM1 message. Since antisense mRNA, in general, has a short half-life, this weak signal is expected. AdCAM101 showed no C-CAM1 protein expression, however. These results indicate that the sense recombinant adenovirus (AdCAM902) is capable of expressing C-CAM1. From the time course study, a continuous elevation of C-CAM1 mRNA was detected in viral-infected PC-3 cells 14 days after infection. Similarly, the C-CAM1 proteins were still detectable 20 days after infection. Since the viral genome was not detected 10 days after infection, these data suggest that the prolonged C-CAM1
expression in sense recombinant virus-infected cells may be due to the high stability of C-CAM1 protein.

**EXAMPLE VII - Tumor Suppressing Effect of C-CAM1 Recombinant Adenovirus**

**Material and Methods**

1. **Measurement of PC-3 tumor growth by in vivo administration of recombinant adenovirus**

To determine the efficacy of C-CAM1 adenovirus on the growth of PC-3 tumors, the inventors injected $1 \times 10^6$ cells/site at four sites in the flanks of 8- to 10-week-old male nude mice subcutaneously. Once the tumor became palpable, 50 μl of either recombinant adenovirus or buffer control (Phosphate-buffered saline + 10% glycerol) were injected near the tumor area (i.e., subcutaneously above the tumors). The change in tumor volume was measured weekly and calculated using the formula described previously in Example III.

**Results**

Previous results, in Example II, indicated that increased C-CAM1 expression in PC-3 cells reduces the *in vitro* growth of this cell line. Based on the *in vitro* time course of C-CAM expression in virus-infected cells (Example VI), the inventors tested the therapeutic efficacy of C-CAM1 on preexisting PC-3 tumors by injecting (50 μl) of either sense or antisense C-CAM1-adenovirus or buffer control into the area near the tumor mass. The growth of tumors was then monitored weekly. As shown in FIG. 9, the growth of PC-3-induced tumors was suppressed by C-CAM1 adenovirus treatment for at least a 3-week period. At week 6, the inventors observed that tumors regained the growth activity. The second dose (50 μl) of virus delivered at week 7 was able to continue tumor suppression for at least a 2-week period (FIG. 9). In the same study, during the first few weeks of viral injection, the inventors observed a small degree of tumor inhibition by antisense (AdCAM101) adenovirus treatment.
suggesting that the virus itself may cause some toxic effect on cells since the titer of AdCAM101 was 10-fold higher than that of AdCAM902. However, from the AdCAM101-treated group, the growth of PC-3 induced tumors eventually resumed and the size of tumors were similar to that of the control (FIG. 9).

Since C-CAM1 adenovirus can suppress the growth of small tumors, the inventors further examined the efficacy of AdCAM902 treatment on larger tumors, ranging from 40 mm$^3$ to 75 mm$^3$, which represent more heterogenous tumors. Based on results in previous examples, the inventors estimated that the effective viral dosage needed to increase at least 3-fold in order to inhibit the growth of larger tumors.

However, it is difficult to deliver such a large volume (e.g., 150 μl) to tumors at once; therefore, the viruses were equally divided into three dosages and delivered every week for 3 consecutive weeks. In this study, the inventors observed that AdCAM902 (FIG. 10C), but not control (FIG. 10A) or AdCAM101 (FIG. 10B), was still able to suppress the growth of PC-3 tumors for at least 5 weeks starting from the first viral injection (time 0). In some cases, however, AdCAM902 failed to suppress the tumor growth if the tumor size reached more than 100 mm$^3$.

EXAMPLE VIII - Construction and Analysis of Recombinant Adenoviruses

This example shows the construction and analysis of recombinant adenoviruses containing a C-CAM1 cDNA and the effects of C-CAM1 expression on the in vivo tumorigenicity of MDA-MB-468 breast cancer cells.

A. Materials and Methods

1. Construction of recombinant adenoviruses

To study the effects of C-CAM1 expression on the in vivo tumorigenicity of the breast cancer cell line MDA-MB-468, a series of adenovirus recombinants were made, as shown in FIG. 11, containing wild-type, anti-sense, or mutant C-CAM cDNA. Recombinant adenoviruses containing the full-length wild-type C-CAM1 cDNA in the sense (AdCAM1) and antisense (AdCAM1-AS) orientations were constructed as described previously (Kleinerman et al., 1995). To construct the
AdCAM1-ΔD1 virus, a 1.5-kb HindIII-NotI fragment containing the C-CAM1 cDNA lacking the first Ig domain was isolated from pCR1000-ΔD1 (Cheung et al., 1993a) and inserted into an adenoviral shuttle vector, XCMV, to generate the plasmid XCMV-CAM1-ΔD1. The recombinant adenovirus AdCAM1-ΔD1 was generated by co-transfection of the plasmids XCMV-CAM1-ΔD1 and pJM17, a vector that contains the adenovirus genome, into the human embryonal kidney cell line 293 as described previously (Kleinerman et al., 1995).

To construct the AdCAM-H458 virus, the CAM1-H458 cDNA fragment with flanking HindIII-NotI sites was generated by PCR™ amplification with oligo 42 (GTGACAAAGCTTATGGAGCTAGCCTCGGCTCGTCTC) and oligo 43 (GCGGCGCCGTGCACGGATATCGATAAGGTGATATC) as primers (the HindIII and NotI sites are underlined) and pSK-H458 (Lin et al., 1995) as the template. The 1.6 kb product was subcloned into pCRII to yield pCRII-Adeno-H458. The DNA fragment coding for CAM1-H458 was isolated from pCRII-Adeno-H458 by digestion with HindIII and NotI, and the fragment was inserted into the adenoviral shuttle vector XCMV at the HindIII-NotI site to generate XCMV-CAM1-H458. AdCAM-H458 was generated by co-transfection as described above.

To construct the AdCAM1-G454 virus, a kinased HindIII recognition sequence was inserted at the SmaI site of pSK-IL60, which contains the sequence for CAM1-G454 (also called C-CAM3) (Cheung et al., 1993c). The 1.5 kb CAM1-G454 cDNA fragment was excised from the plasmid with HindIII and XmnI and inserted into pBluescript pBSK (Stratagene, La Jolla, CA) to generate pSK-IL60-Adeno. A 1.5 kb HindIII-NotI fragment containing the CAM1-G454 cDNA, which lacks the C-terminal 65 amino acids of C-CAM1, was isolated from pSK-IL60-Adeno and inserted into the adenovirus shuttle vector XCMV at the HindIII-NotI site to generate XCMV-CAM1-G454. AdCAM-G454 was generated by co-transfection as described above.
2. Detection of C-CAM protein by FACS analysis.

MDA-MB-468 cells were infected with recombinant adenovirus at various virus to cell (V/C) ratios. After 24 hr, fluorescence staining of C-CAM expressing cells was performed with an anti-C-CAM polyclonal antibody (Ab669) and fluorescein isothiocyanate-conjugated secondary antibodies as described previously (Cheung et al., 1993a). FACS analysis was performed as described previously (Hsieh et al., 1995).

3. Immunoprecipitation and western blotting.

To analyze the C-CAM1 proteins expressed in MDA-MB-468 cells, aliquots of cell lysate from infected and uninfected cells were boiled in sodium dodecyl sulfate (SDS) sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Western immunoblotting with Ab669 was performed as previously described (Lin et al., 1995). To immunoprecipitate the C-CAM1 proteins expressed in MDA-MB-468 cells, aliquots of cell lysate (50 μl) from infected MDA-MB-468 cells were mixed with an equal volume of denaturing buffer (1 mM EDTA, 1% SDS, and 1 mM dithiothreitol, pH 8.0) and boiled for 5 min. After removal of insoluble material by centrifugation, the supernatants were diluted with immunoprecipitation buffer (150 mM NaCl, 1 mM EDTA, 0.5% deoxycholate, 1% Nonidet P-40, and 25 mM Tris-HCl, pH 7.5) to a final volume of 1 ml and incubated with 3 μl of antipeptide antibody and 20 μl of protein G-Sepharose (Pharmacia Inc., Piscataway, NJ) for 16 hr at 4°C with constant mixing. The immunoprecipitates were washed once with 0.8 ml of immunoprecipitation buffer containing 0.1 ml of saturated NaCl, then with 0.8 ml of immunoprecipitation buffer containing 0.1% SDS, and finally with 0.8 ml of immunoprecipitation buffer alone. The materials bound to protein G-Sepharose were eluted by boiling the protein G-Sepharose in SDS sample buffer. Western immunoblotting with Ab669 was performed as previously described (Lin et al., 1995).
4. Tumorigenicity in nude mice

MDA-MB-468 cells were infected with recombinant adenovirus at V/C ratio of 10:1. Twenty-four hr after infection, the cells were removed from the plates by trypsin digestion and washed twice with phosphate-buffered saline. One million cells were then injected subcutaneously into each of six sites in the left and right flanks of nude mouse and tumor growth was monitored weekly. Tumor volume was estimated by the method of Rockwell et al. (1972).

B. Results

To study the effects of C-CAM1 expression on the in vivo tumorigenicity of the breast cancer cell line MDA-MB-468, a full-length C-CAM1 cDNA was inserted into an adenoviral vector to generate a C-CAM1 recombinant adenovirus (AdCAM1) as a gene transfer vehicle. The breast-tumor cell line MDA-MB-468 was highly tumorigenic when injected into nude mice, displaying tumor growth in 16/18 animals, or 89% (Table 7). MDA-MB-468 cells infected with recombinant wild-type or mutant adenoviruses displayed varying incidences of tumor growth: AdCAM1, 2/18 (11%); AdCAM1-AS, 12/18 (67%); AdCAM1-ΔD1, 3/18 (17%), AdCAM1-H458, 14/18 (78%); AdCAM1-G454, 15/18 (83%) (Table 7). On western immunobLOTS with a polyclonal antibody against C-CAM (Ab669), C-CAM protein expression was not detectable in MDA-MB-468 cells. This result is consistent with those observed in hepatoma and prostate cancer in that C-CAM is down-regulated in these tumors. To determine the expression efficiency of C-CAM1 in MDA-MB-468 cells, the inventors infected them with AdCAM1 at various virus to cell (V/C) ratios. Because C-CAM1 is an integral transmembrane protein, the efficiency of C-CAM expression was determined by fluorescence-activated cell sorting (FACS) with a polyclonal antibody against C-CAM1 (Ab669) (Kleinerman et al., 1995). About 45%, 56%, 69%, 73% and 86% of cells expressed C-CAM1 on their surfaces when infected with AdCAM1 at the V/C ratios of 5:1, 10:1, 20:1, 30:1, and 50:1, respectively, for 24 hr. In addition, the total amounts of protein expressed, as reflected by the increase in
fluorescence intensity, also increased as the V/C ratio increased. These results suggest that MDA-MB-468 cells are susceptible to adenoviral infection and can efficiently express and process the protein product of the C-CAM1 recombinant adenovirus.

Table 7. Tumor incidence induced by C-CAM1 and C-CAM1 mutant recombinant adenovirus infection of MDA-MB-468 cells

<table>
<thead>
<tr>
<th>Adenovirus Infection</th>
<th>Tumor Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (no infection)</td>
<td>16/18 (89)</td>
</tr>
<tr>
<td>AdCAM1-AS</td>
<td>12/18 (67)</td>
</tr>
<tr>
<td>AdCAM1</td>
<td>2/18 (11)</td>
</tr>
<tr>
<td>AdCAM1-ΔD1</td>
<td>3/18 (17)</td>
</tr>
<tr>
<td>AdCAM1-H458</td>
<td>14/18 (78)</td>
</tr>
<tr>
<td>AdCAM1-G454</td>
<td>15/18 (83)</td>
</tr>
</tbody>
</table>

Tumors larger than 10mm³ were considered to be positive.

Although the size of C-CAM1 is predicted to be 53 kDa based on its cDNA sequence (Lin and Guidotti, 1989; Lin et al., 1991), the proteins expressed from MDA-MB-468 cells after infection with AdC-CAM1 had a relative mass of 120 kDa, suggesting that the C-CAM1 proteins were glycosylated. In addition to showing the 120 kDa protein, a western blot also showed an approximately 85 kDa protein. It is possible that the 85 kDa protein represents a C-CAM1 glycosylation variant, because western analysis of AdCAM1-infected cell lysate after treatment with peptide N-glycosidase F revealed only one protein band of about 53 kDa. Similar protein species were observed when the cells were infected with C-CAM1 mutant recombinant viruses. The AdCAM1-ΔD1 mutant proteins were about 20 kDa smaller than those of AdCAM1, perhaps because the first Ig domain contains three potential N-linked glycosylation sites (Lin et al., 1991), and because the 102 amino acids coding for the first Ig domain were deleted. On the other hand, deletions in the
cytoplasmic domain (in AdCAM1-H458 and AdCAM1-H454) decreased the masses of the proteins by only about ~7 kDa. The identities of the mutant proteins were further confirmed by immunoprecipitation with antipeptide antibody against the cytoplasmic domain (anti-C3) (Lin et al., 1991). AdCAM1 and AdCAM1-ΔD1 proteins were immunoprecipitated with antibody anti-C3, but AdCAM1-H458 and AdCAM1-G454 proteins were not, consistent with the deletion of the cytoplasmic domain.

EXAMPLE IX - Cell adhesion assay of recombinant adenovirus infected MDA-MB-468 cells.

This example shows the cell-adhesive activity of breast cancer cells infected with recombinant adenoviruses containing a wild type or mutant C-CAM1 cDNA.

A. Materials and Methods
   1. Cell adhesion assay

MDA-MB-468 cells were infected with AdCAM1 or its mutants. Twenty-four hr after infection, the cells were trypsinized from the tissue culture plates and resuspended in phosphate buffered saline. These cell suspensions (1 ml) were mixed gently at room temperature to allow the formation of cell aggregates. The number of single cells remaining in suspension was determined with a hemacytometer after 5 hr. The extent of cell aggregation was expressed as the decrease in the percentages of single cells.

B. Results

To test the cell-adhesive activity of these various mutants, cell-aggregation assays were performed. MDA-MB-468 cells were infected with recombinant adenovirus at V/C ratio of 10:1. At 24 hr after infection, the cells were trypsinized, and their ability to form aggregates was monitored. As shown in FIG. 13, MDA-MB-468 cells expressing wild-type C-CAM1 protein were able to aggregate, as evidenced by the decrease in the percentage of single cells (about 50%), as compared with cells
infected with control antisense adenovirus. Expression of AdCAM1-ΔD1 did not confer cell adhesion (FIG. 13), which is consistent with the inventors' observations in the insect cell system (Cheung et al., 1993a). Both AdCAM1-H458 and AdCAM1-G454 were able to cause MDA-MB-468 cells to aggregate. These results are in contrast to those of the inventors' insect cell studies, in which AdCAM1-G454 had no cell-adhesion activity (Cheung et al., 1993c). These differences may be a consequence of the different cell types used for expression or the amount of protein expressed per cell.

**EXAMPLE X - C-CAM1 cytoplasmic domain involved in tumor suppression**

This example shows the effects of expression of C-CAM1 mutants on the *in vivo* tumorigenicity of MDA-MB-468 cells.

**A. Materials and Methods**

1. *Tumorigenicity in nude mice*

MDA-MB-468 cells were infected with recombinant adenovirus at V/C ratio of 10:1. Twenty-four hr after infection, the cells were removed from the plates by trypsin digestion and washed twice with phosphate-buffered saline. One million cells were then injected subcutaneously into each of six sites in the left and right flanks of nude mouse and tumor growth was monitored weekly. Tumor volume was estimated by the method of Rockwell et al. (1972).

**B. Results**

The tumor-suppressive activities of the C-CAM1 mutants were studied by infecting MDA-MB-468 cells with the recombinant adenoviruses at a V/C ratio of 10:1. The efficiency of infection was determined by flow cytometry at 24 hr after infection. About 45% of the cells infected with AdCAM1, AdCAM1-ΔD1, or AdCAM1-H458, were positive for C-CAM staining. About 80% of the cells infected with AdCAM1-G454 were positive for C-CAM staining. The reason for the higher efficiency of AdCAM1-G454 expression is not clear. The infected cells were then
injected subcutaneously into nude mice, and tumor growth was monitored weekly. AdCAM1-ΔD1 infection reduced both the tumor incidence and size in a fashion similar to that of infection with AdCAM1 (Table 7 and FIG. 12 A and B). These results suggest that the adhesion activity of C-CAM1 is not required for C-CAM1-mediated tumor suppression. On the other hand, C-CAM1 mutants with deletions in their cytoplasmic domains (AdCAM1-H458 and AdCAM1-G454) did not suppress tumorigenicity, producing a tumor incidence and volume similar to those of controls (Table 7 and FIG. 12 A and B). These results strongly suggest that the cytoplasmic domain of C-CAM1 is involved in C-CAM1-mediated tumor suppression.
REFERENCES

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.


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Giancotti and Ruoslahti, "Elevated levels of the \(\alpha_5\beta_1\) fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells," *Cell*, 60:849-859, 1990.


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5


15


20


25


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   (B) COMPUTER: IBM PC compatible
   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
   (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vii) CURRENT APPLICATION DATA:
   (A) APPLICATION NUMBER: Unknown
   (B) FILING DATE: Concurrently Herewith
   (C) CLASSIFICATION: Unknown

40 (viii) PRIOR APPLICATION DATA:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1921 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "DNA"
(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 519 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Gly Leu Leu Leu Thr Ala Ser Leu Leu Thr Tyr Trp Ser Pro Leu Thr
20  25  30
Thr Ala Gln Val Thr Val Asp Ala Val Pro Pro Asn Val Val Glu Glu
35  40  45
Lys Ser Val Leu Leu Leu Ala His Asn Leu Pro Gln Glu Phe Gln Val
50  55  60
Phe Tyr Trp Tyr Lys Gly Thr Thr Leu Asn Pro Asp Ser Glu Ile Ala
65  70  75  80
Arg Tyr Ile Arg Ser Asp Asn Met Ser Lys Thr Gly Pro Ala Tyr Ser
85  90  95
Gly Arg Glu Thr Ile Tyr Ser Asn Gly Ser Leu Phe Phe Gln Asn Val
100 105 110
Asn Lys Thr Asp Glu Arg Ala Tyr Thr Leu Ser Val Phe Asp Gln Gln
115 120 125
Phe Asn Pro Ile Gln Thr Ser Val Gln Phe Arg Val Tyr Pro Ala Leu
130 135 140
Gln Lys Pro Asn Val Thr Gly Asn Ser Asn Ser Pro Met Glu Gly Glu
145 150 155 160
Pro Phe Val Ser Leu Met Cys Glu Pro Tyr Thr Asn Thr Ser Tyr
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Leu Trp Ser Arg Asn Gly Ser Leu Ser Glu Gly Asp Arg Val Thr
180 185 190
Phe Ser Glu Gly Asn Arg Thr Leu Thr Leu Leu Asn Val Arg Arg Thr
195 200 205
Asp Lys Gly Tyr Tyr Glu Cys Glu Ala Arg Asn Pro Ala Thr Phe Asn
210 215 220
Arg Ser Asp Pro Phe Asn Leu Asp Val Ile Tyr Gly Pro Asp Ala Pro
225 230 235 240
Val Ile Ser Pro Pro Asp Ile Tyr Leu His Glu Gly Ser Asn Leu Asn
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Ser Val Ala Gly Val Ala Leu Ala Ala Ala Tyr Phe Leu Tyr
435 440 445
Ser Arg Lys Thr Gly Gly Gly Ser Asp His Arg Asp Leu Thr Glu His
450 455 460
Lys Pro Ser Thr Ser Ser His Asn Leu Gly Pro Ser Asp Asp Ser Pro
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Asn Lys Val Asp Asp Val Ser Tyr Ser Val Leu Asn Phe Asn Ala Gln
485 490 495
Gln Ser Lys Arg Pro Thr Ser Ala Ser Ser Ser Pro Thr Glu Thr Val
500  505  510

Tyr Ser Val Val Lys Lys Lys
5  515
CLAIMS:

1. An expression construct comprising a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, wherein said nucleic acid is under transcriptional control of said promoter.

2. The expression construct according to claim 1, further comprising a polyadenylation signal.

3. The expression construct according to claim 1, further comprising a selectable marker.

4. The expression construct according to claim 1, wherein said nucleic acid is a cDNA.

5. The expression construct according to claim 1, wherein said nucleic acid is a genomic DNA.

6. The expression construct according to claim 1, wherein said expression construct is an adenovirus.

7. The expression construct according to claim 6, wherein said adenovirus lacks at least a portion of the E1 region.
8. The expression construct according to claim 1, wherein said nucleic acid is positioned in a sense orientation with respect to said promoter.

9. The expression construct according to claim 1, wherein said nucleic acid is positioned in an antisense orientation with respect to said promoter.

10. A pharmaceutical composition comprising (i) an expression construct comprising a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, wherein said nucleic acid is under transcriptional control of said promoter and (ii) a pharmaceutically acceptable buffer, solvent or diluent.

11. A method for restoring C-CAM function in a cell that lacks C-CAM function comprising the steps of:

   providing an expression construct comprising a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, said nucleic acid under the control of said promoter and nucleic acid positioned in a sense orientation to said promoter; and

   contacting said expression construct with said cell that lacks C-CAM function.

12. The method of claim 11, wherein said cell is a transformed cell and said contacting reverses said transformed phenotype.

13. The method of claim 12, wherein said cell is a prostate or tumor cell.
14. The method of claim 12, wherein said cell is a bladder tumor cell.

15. The method of claim 12, wherein said cell is a breast tumor cell.

16. The method of claim 11, wherein said expression construct is an adenovirus.

17. A method for inhibiting C-CAM function in a cell comprising the steps of:

   providing an expression construct comprising a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, said nucleic acid under the control of said promoter and positioned in an antisense orientation to said promoter; and

   contacting said expression construct with said cell.

18. The method of claim 17, wherein said cell is transformed by said contacting.

19. The method of claim 17, wherein said expression construct is an adenovirus.
20. A method of treating a mammal with cancer comprising:

providing a pharmaceutical composition comprising (a) an adenovirus comprising a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, said nucleic acid under the control of said promoter and positioned in a sense orientation to said promoter, and (b) a pharmaceutically acceptable buffer, solvent or diluent; and

administering said pharmaceutical composition to said mammal.

21. The method according to claim 20, wherein said mammal is a human.

22. The method according to claim 20, wherein said administering is via intravenous injection.

23. The method according to claim 20, wherein said administering is via orthotopic injection.

24. The method according to claim 20, wherein said cancer is prostate cancer.

25. The method according to claim 20, wherein said cancer is bladder cancer.
26. The method according to claim 20, wherein said cancer is breast cancer.

27. A kit comprising, in suitable container means, an expression construct comprising a promoter functional in eukaryotic cells and a nucleic acid encoding a C-CAM, wherein said nucleic acid is under transcriptional control of said promoter, and a pharmaceutically acceptable buffer, solvent or diluent.

28. A method for detecting cancer cells in a sample by detecting a nucleic acid encoding a C-CAM.

29. A method for detecting cancer cells in a sample by detecting a C-CAM.
FIG. 1
FIG. 3
FIG. 6
FIG. 10C

AdCAM902

Tumor Volume (cubic mm)

Treatment (Weeks)
C-CAM1: sig | D1 | D2 | D3 | D4 | LTM | Cyto (71 a.a.)
CAM1-ΔD1: sig | D2 | D3 | D4 | LTM | Cyto (71 a.a.)
CAM1-H458: sig | D1 | D2 | D3 | D4 | LTM | Cyto (10 a.a.)
CAM1-G454: sig | D1 | D2 | D3 | D4 | LTM | Cyto (6 a.a.)

FIG. 11
FIG. 12A
FIG. 12B
FIG. 13

% Single Cells in Aggregation Assay
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

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According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

Date of the actual completion of the international search

3 October 1996

Date of mailing of the international search report

28.10.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 TV Rijswijk Tdl. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016

Authorized officer

Chambonnet, F
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<td>FASEB JOURNAL FOR EXPERIMENTAL BIOLOGY, vol. 9, no. 3, 9 - 13 April 1995, BETHESDA, MD US, page 401 XP002015051 COMEGYS, M. ET AL.: &quot;Rat strain and cell-type specific expression of C-CAM 2&quot; see abstract &amp; Experimental biology 95, Atlanta, USA, April 9-13 1995 Poster discussion</td>
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<td>WO,A,95 02697 (RHONE-POULENC RORER) 26 January 1995 see claims 1-5,13,14,17,28-30</td>
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Relevant to claim No.

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# INTERNATIONAL SEARCH REPORT

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **X** Claims Nos.:  
   Because they relate to subject matter not required to be searched by this Authority, namely: 
   **Remark:** Although claims 20-26 and partially claims 11-19, as far as they concern an in vivo method, are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. ☐ Claims Nos.:  
   Because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:  
   Because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.
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