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(54) Title: CD44 EXPRESSION IN SMOOTH MUSCLE CELLS

(57) Abstract

Disclosed are methods of treating arteriosclerosis or vascular restenosis by inhibiting CD44-mediated smooth muscle migration or proliferation as well as methods of screening for the identification of compounds capable of inhibiting the expression of CD44, inhibiting the CD44-mediated migration of smooth muscle cells, and inhibiting the CD44-mediated proliferation of smooth muscle cells.

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CD44 EXPRESSION IN SMOOTH MUSCLE CELLS

Background of the Invention

This invention relates to the prevention and treatment of arteriosclerosis.

CD44 (Pgp-1, Hermes-3, HCAM, ECMR III) is a widely expressed glycoprotein with a molecular weight of 85 to 90 kDa (Haynes et al., 1989, Immunol. Today 10:423-428). Immunological studies have shown that CD44 is involved in a diverse range of biological functions, such as lymphocyte binding to high endothelial venules (Jalkanen et al., 1987, J. Cell. Biol. 105:983-990), lymphopoiesis (Miyake et al., 1990, J. Exp. Med. 171:477-488) and activation of leukocytes (Webb et al., 1990, Science 249:1295-1297). CD44 has also been shown to play a role in extracellular matrix binding, cell migration, lymphocyte activation, lymphocyte homing, and proliferation of bronchial smooth muscle cells (Herrlich et al., Immunology today, 14(8):395-399, 1993; Lesley et al., Immunology, 54:271-335, 1993; Lazzar et al., Journal of Experimental Medicine, 180:807-816, 1994). A splice variant of CD44, CD44-V6, has been shown to play a role in tumor cell metastasis (Gunthert et al., Cell, 65:13-24, 1991). CD44 cDNA sequence has revealed a domain of some 90 amino acids near the N-terminus bearing a significant similarity to the link and core proteins in proteoglycan (Goldstein et al., 1989, Cell 56:1063-1072), leading to the establishment of hyaluronate, a major component of the extracellular matrix, as a ligand for CD44 (Miyake et al., 1990, J. Exp. Med. 172:69-75; Aruffo et al., 1990, Cell 61:1303-1313). Fibronectin and

collagen type I and VI have also been shown to interact with CD44 (Carter et al., 1988, J. Biol. Chem. 263:4193-4201).

CD44 is a widely distributed heterogenous population of cell surface adhesion molecules. The CD44 gene has twenty exons, ten of which encode the sequence for standard CD44. An additional ten variable exons are inserted by alternative splicing.

Although the standard 85 kDa CD44 is broadly expressed by many different types of cells, the expression of CD44 variants is rather limited. Arch et al. have demonstrated a transient expression of a CD44 variant, V6, in leukocytes from animals after allogeneic immunization (Arch et al, 1992, Science 257:682-685), indicating a possible role for this variant in mediating leukocyte trafficking.

Initially, the heterogeneity of CD44 was thought to be due to post-translation modification, especially the addition of chondroitin sulfate, which gives rise to a higher molecular weight form of about 200 kDa (Jalkanen et al., 1988, J. Immunol. 141:1615-1623). The finding of a protein isoform in rats capable of conferring metastatic potential to nonmetastatic cells established a new role for CD44, namely, regulating cell migration (Gunthert et al, 1991, Cell 65:13-24).

So far, there are at least 18 different CD44 isoforms identified in various species of animals (Stamenkovic et al., 1991, Embo. J. 10:343-8; He et al., 1992, J. Cell. Biol. 119:1711-1719; Jackson et al., 1992, J. Biol. Chem. 267:4732-4739). But the functional significance of these isoforms is still unknown.

Summary of the Invention

It has now been discovered that smooth muscle cells that have been activated either by being placed in

cell culture or in response to mechanical injury, in an experimental lesion analogous to angioplasty, express the cell surface antigen, CD44. CD44 is not expressed in normal, resting smooth muscle cells.

The smooth muscle cell is a major participant in arteriosclerosis or in the stenotic lesions that occur after angioplasty or bypass surgery of the coronary vessels. Thus, CD44 may provide a unique target for intervention in either arteriosclerosis or restenosis.

Accordingly, the invention features a method of treating arteriosclerosis (the abnormal hardening or thickening of arterial walls) or vascular restenosis (the narrowing or constriction of blood vessel walls) in an animal by inhibiting the expression of CD44 on smooth muscle cells. By the term "expression" is meant the transcription of the gene encoding CD44, translation of the transcript into protein product, translocation of the protein product to the cell membrane and appropriate anchoring of the protein product in the cell membrane.

In one embodiment, expression of CD44 may be inhibited by introducing CD44 antisense DNA into smooth muscle cells. By the term "antisense DNA" is meant a nucleic acid which binds to the coding strand of DNA resulting in the prevention of transcription. Preferably, the antisense DNA is complementary to the coding strand of DNA of CD44. Other methods of inhibiting expression of CD44 may also be used, e.g., inhibiting translation of the RNA transcript, inhibiting translocation of the protein product. In yet another embodiment, expression of the CD44-V6 isoform of CD44 is inhibited. In another embodiment, smooth muscle cell expression of CD44 is upregulated as a result of blood vessel injury.

In a second aspect, the invention features a method of decreasing migration of a smooth muscle cell by

inhibiting the expression of CD44. As described above, expression of CD44 or the CD44-V6 isoform, which may have been upregulated as a result of blood vessel injury, may be inhibited by introducing CD44 antisense DNA into the smooth muscle cells.

In a third aspect, the invention features a method of decreasing proliferation of a smooth muscle cell by inhibiting the expression of CD44.

In a fourth aspect, a method of decreasing smooth muscle cell migration is featured. According to this method, a smooth muscle cell is contacted with a compound which blocks the binding of the cell to a component of the extracellular matrix. In one embodiment, the compound is a CD44-specific antibody or an antigen-binding fragment thereof, e.g., an antibody or antigen-binding fragment which binds to the V6 domain of CD44. The compound may also be a soluble fragment of CD44, e.g., a fragment of CD44 which includes the V6 domain of the protein. In another embodiment, the compound binds to the extracellular domain of CD44. The compound may be hyaluronic acid, fibronectin, or any compound with the binding properties of hyaluronic acid or fibronectin. In yet another embodiment, the compound may be a soluble fragment of the cytoplasmic domain of CD44 or any compound which inhibits CD44-mediated signal transduction.

A fifth aspect of the invention features a method of decreasing migration of a smooth muscle cell by contacting a smooth muscle cell with a compound which blocks the binding of the cell to a second cell.

A sixth aspect of the invention defines a method of decreasing proliferation of a smooth muscle cell by contacting the smooth muscle cell with a compound which blocks the binding of the cell to a component of the extracellular matrix.

In a seventh aspect, the invention features a method of decreasing proliferation of a smooth muscle cell by contacting the smooth muscle cell with a compound inhibits CD44-mediated signal transduction, e.g., a soluble fragment of the cytoplasmic domain of CD44.

In an eight aspect, the invention encompasses a method of decreasing proliferation of a smooth muscle cell by contacting a smooth muscle cell with a compound which blocks the binding of the cell to a second cell.

In a ninth aspect of the invention, a method of screening candidate compounds to identify a compound capable of inhibiting CD44-mediated smooth muscle cell migration is featured. The screening assay includes the steps of providing a smooth muscle cell bearing CD44 on its surface; contacting the smooth muscle cell with a candidate compound; and, determining the amount of smooth muscle cell migration. A decrease in migration of the smooth muscle cell in the presence of a candidate compound compared to the amount in the absence of a candidate compound indicates that the candidate compound inhibits CD44-mediated smooth muscle cell migration.

The tenth aspect, the invention features a method of screening candidate compounds to identify a compound capable of inhibiting CD44-mediated smooth muscle cell proliferation. The screening assay includes the steps of providing a smooth muscle cell bearing CD44 on its surface; contacting the smooth muscle cell with a candidate compound; and, determining the amount of proliferation of the smooth muscle cell. A decrease in the amount of proliferation in the presence of a candidate compound compared to the amount in the absence of a candidate compound indicates that the candidate compound inhibits CD44-mediated smooth muscle cell proliferation.

In a final aspect, the invention features a method of screening candidate compounds to identify compounds capable of inhibiting expression of CD44. The screening method of the invention includes the following steps: providing an activated smooth muscle cell; contacting the smooth muscle cell with a candidate compound; and determining the amount of CD44 expression in the smooth muscle cell. A decrease in the amount of CD44 expression in cells exposed to the candidate compound compared to the amount of expression in cells in the absence of compound indicates that the compound inhibits expression of CD44 in smooth muscle cells.

Other features and advantages of the invention will be apparent from the following detailed description and from the claims.

Brief Description of the Drawings

Fig. 1 is a diagram comparing the structure of the V6 isoform of CD44 expressed on smooth muscle cell to the structure of standard CD44.

Fig. 2 is a diagram showing the structure and post-translational modification of proteoglycan CD44. CD44 is shown anchored in the cell membrane.

Fig. 3 is a histogram showing expression of CD44 on the surface of smooth muscle cells.

Fig. 4 is a histogram showing the expression of the CD44-V6 variant on rat smooth muscle cells (RASMC).

Fig. 5 is histogram showing FACS analysis of surface antigens of human aortic smooth muscle cells (HASMC) and RASMC.

Fig. 6A is a histogram showing cell surface expresssion of CD44 on RASMC.

Fig. 6B is a histogram showing cell surface expresssion of β_1 on RASMC.

Fig. 6C is a histogram showing cell surface expresssion of CD44 V6 on RASMC.

Fig. 6C is a histogram showing cell surface expresssion of β_3 on RASMC.

Fig. 7 is a photograph of a Northern blot analysis showing CD44 expression in quiescent smooth muscle cells compared to activated smooth muscle cells.

Fig. 8A is a bar graph showing a comparison of CD44 expression in RASMC grown on plastic in 10% FCS (growing and confluent) to CD44 expression in RASMC grown on matrigel

Fig. 8B is a photograph of a Northern blot showing a comparison of CD44 expression in RASMC grown on plastic in 10% FCS (growing and confluent) to CD44 expression in RASMC grown on matrigel.

Fig. 9A is a photograph of a Northern blot showing CD44-V6 expression in carotid artery tissue after carotid artery balloon injury; 20 μg of RNA was loaded per lane.

Fig. 9B is a bar graph showing CD44-V6 expression in carotid artery tissue after carotid artery balloon injury.

Fig. 10A is a Northern blot showing CD44 expression in carotid artery tissue after carotid artery injury; 7.5 μg of RNA was loaded per lane.

Fig. 10B is a bar graph showing CD44-V6 expression after carotid artery balloon injury.

Fig. 11A is a bar graph showing proliferation of HASMC to hyaluronate.

Fig. 11B is a bar graph showing proliferation of RASMC to hyaluronate.

Fig. 12A is a bar graph showing proliferation of HASMC to antibody.

Fig. 12B is a bar graph showing proliferation of RASMC to antibody.

Detailed Description

Cell Culture

RASMC were harvested from the thoracic aorta of male Sprague-Dawley rats (200-250 g) by enzymatic dissociation. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 mM Hepes (pH 7.4) in a 37° C, 5% CO_2, humidified incubator. Cells from passage 5-8 were used in the experiments. HASMC (Clonetics, San Diego) were grown in M199 medium (GIBCO) containing 20% FCS, penicillin (100 units/ml), streptomycin (100 μg/ml), and 25 mM Hepes (pH 7.4). Cells from passages 8-10 were used.

To promote differentiation, RASMC were grown *in vitro* on matrigel (Collaborative Research) according to the manufacturer's instructions. Cells (2×10^6) were evenly plated on the dishes and examined for morphological changes after 24 hours.

[^{3}H]-Thymidine Incorporation

RASMC and HASMC in 24 well plates were made quiescent by incubation in DMEM (0.4% CS) and M199 medium (0.4% FCS), respectively, for 72 hours before addition of hyaluronic acid (HA) (Anika Chemical Company, Boston, MA) or monoclonal antibody, e.g., OX-49 (Pharmingen, San Diego, CA), OX-50 (Biosource International, Camarillo, CA), NIH44 (National Institutes of Health). During the last two hours of a 24 hour incubation, cells were labeled with methyl-[^{3}H]-thymidine (DuPont/NEN) at 1 μCi/ml (1 μCi=37 kBq). After labeling, cells were washed with Dulbecco's phosphate-buffered saline and fixed in cold 10% trichloroacetic acid, then washed with 95% ethanol. Incorporated [^{3}H]-thymidine was extracted in 0.2N NaOH and measured in a liquid scintillation counter.

Values were expressed as the mean ± SEM from 6 wells from two separate experiments. Statistical analysis was performed by the Kruskal-Wallis test and significance accepted at $P<0.05$.

Amplification of CD44 and CD44-V6 from RASMC

A cDNA fragment from RASMC RNA was amplified by the reverse transcription polymerase chain reaction. Primers were designed using the published sequence of the rat CD44 and CD44-V6 sequence. The following primers were used to amplify a 1228 base pair fragment of CD44: forward primer 5′ AGCCAGTGACAGGTTCCATT 3′(SEQ ID NO:1) and reverse primer 5′ TGTTGTGTCTTTTCAAGTTA 3′ (SEQ ID NO:2). For CD44-V6, the forward primer 5′ GGCGGATCCTAATAGCACAACAG 3′ (SEQ ID NO:3) and reverse primer 5′ GGCGGATCCTTCTGTCACATGGGAG 3′ (SEQ ID NO:4) were used to amplify a 108 base pair fragment. The polymerase chain fragment was then subcloned and sequenced by the dideoxy chain termination method known in the art. The sequence of the two fragments were identical to the known sequence (Gunthert, 1991, CELL 65:13-24).

RNA Extraction and RNA Blot Analysis

Rats subjected to balloon carotid injury were obtained at various time points from Zivic-Miller Company. Rats were anesthetized using chloral hydrate, and the injured carotid artery isolated. Total RNA was extracted using the RNA-Zol method (Cinna/Ciotecx Laboratories International). Total RNA from cultured cells was obtained by guanidinium isothiocyanate extraction and centrifuged through cesium chloride. The RNA was fractionated on a 1.3% formaldehyde-agarose gel and transferred to nitrocellulose filters. The filters were hybridized with a randomly primed ^{32}P-labeled CD44 or CD44-V6 probes. The hybridized filters were washed in 30

mM sodium chloride, 3 mM sodium citrate, and 0.1% sodium dodecyl sulfate solution at 55° C and autoradiographed with Kodak XAR film at -80° C for 12-48 hours or stored on phosphor screens for 12-18 hours. To correct for differences in RNA loading, the filters were washed in a 50% formamide solution at 80° C and rehybridized with a 18S oligonucleotide probe. The filters were scanned, and radioactivity was measured on a PhosphorImager running the ImageQuant software (Molecular Dynamics, Inc., Sunnyvale, CA).

Immunocytochemistry and *in situ* hybridization

Male rats after injury to the carotid artery (Zivic-Miller) were perfused with 4% paraformaldehyde and carotid arteries harvested and embedded in paraffin. Tissue specimens were incubated with primary antibody for 1 hour followed by three washes with PBS. Specimens were then incubated with biotin-conjugated secondary antibody overnight. Primary antibodies used were as follows: Ox-49 (anti-rat CD44, IgG2a) and 1.1 ASML (anti-rat CD44-V6, IgG1).

For *in situ* analysis, carotid specimens were fixed in paraformaldehyde, sucrose dehydrated, and embedded in O.C.T. Five microns sections were obtained. Digoxigenin labeled riboprobes were generated using the cloned cDNA fragments of CD44 and CD44-V6. Hybridization to tissue sections was done at 55°C overnight and washed with 5 × SSC followed by a wash with 2 × SSC with 50% formamide. Specimens were then treated with RNA-ase and blocked with bovine serum albumin (BSA). Tissue labeling was detected using alkaline phosphatase labeled anti-digoxigenin antibody (Boerhinger Manheim, Indianapolis, IN).

Flow cytometry analysis

Rat aortic smooth muscle cells were obtained as described above. Cells were briefly exposed to 0.025%/1.5 mM EDTA, washed and incubated on ice for 30 minutes with various antibodies. Labeled cells were washed three times with DMEM media containing 10% FCS and incubated with FITC-conjugated secondary antibody. Analysis was done on a Beckton Dickinson FACS scanner. Antibodies used were: Ox-49; 1.1 ASML; rabbit anti-rat β_1 integrin antiserum; F11 (anti-rat β3 integrin, IgG1 (Pharmingen).

Expression of the V6 variant of CD44 on RASMC

The involvement of CD44 variants in arterial smooth muscle cell activities was studied using a rat carotid artery injury model. The existence of CD44 variants and their potential in regulating medial smooth muscle cell proliferation and migration was examined. Specifically, the role of CD44 in the migration of smooth muscle cells involved in the formation of lesions characteristic of vascular injury was examined. This disclosure represents the first report of CD44 and its V6 isoform expression on smooth muscle cells. Cell surface expression of CD44 was measured by FACS and tissue staining. Expression of CD44 was confirmed by analysis of CD44-V6 transcripts in cultured smooth muscle cells.

Immunofluorescence studies

Using the well-known method of flow cytometry, smooth muscle cells were found to express high levels of CD44 on their surface (see Figs. 3-5).

In Fig. 3, cultured smooth muscle cells were incubated in the presence and absence of tumor necrosis factor-α (TNF-α), an inflammatory cytokine, followed by

FACS analysis of the cells using an anti-CD44 antibody. The right peak of the histogram represents CD44 expression. The data, which is plotted on a logarithmic scale, clearly indicates that CD44 expression is substantially increased on cells exposed to TNF-α. Cells incubated in the absence of TNF-α were also observed to express CD44, but in amounts much lower than the TNF-α-treated cells. The low level of expression is believed to be due to a basal level of activation of cultured smooth muscle cells.

To further investigate this observation, expression of CD44 on cultured human muscle cells grown in culture media deprived of serum growth factors was compared to the expression on cells grown under optimal conditions, i.e., media supplemented with serum growth factors. Fig. 5 shows that CD44 expression is elevated in cells grown under normal conditions compared to cells grown under suboptimal conditions. CD44 was found to be expressed on cultured human as well as cultured RASMC. These data indicate that CD44 expression is correlated with activation of smooth muscle cells.

Binding of the isoform-specific monoclonal antibody, 1.1ASML to the surface of smooth muscle cells indicated that the CD44 molecule expressed on the surface of these cells was a particular isoform, the V6 variant of CD44 (Fig. 4). Both the V6 isoform and the standard isoform of CD44 were found to be expressed on the surface of cultured smooth muscle cells.

Tissue Staining

CD44 expression was also examined in primary tissues. Rat carotid arteries were collected from normal and balloon injured animals. Tissue samples were stained with anti-CD44 monoclonal antibodies. In contrast to studies using cultured smooth muscle cells, neither CD44

standard form nor the V6 isoform was detectable on normal vessels. However, after mechanical injury of a blood vessel by balloon denudation (angioplasty), CD44-V6 was found to be expressed in both the neointima of the blood vessel and in the media as indicated by immunofluorescence staining. These results indicate that expression of CD44 is increased after injury.

Distribution of CD44 in smooth muscle cells

Using immunofluorescent tissue staining, anti-CD44 monoclonal antibodies were observed to form bright stretches along the edges of the stained cells. This observation suggests that some surface CD44 may be engaged in the adhesion to fibronectin or other substances in the extracellular matrix.

Detection of CD44 transcripts in smooth muscle cells

Smooth muscle cells shown to express CD44-V6 by FACS analysis or immunofluorescent tissue staining were subjected to further analysis. RNA was isolated from cultured smooth muscle cells and tested for the presence of CD44-V6-specific transcripts. The detection of isoform-specific RNA transcripts confirmed the cell surface expression data.

Tissue samples from normal and injured blood vessels were analyzed for CD44 transcription. Competitive PCR revealed a significant increase of CD44-V6 transcript in injured arteries, confirming the tissue staining data described above. Taken together, these data indicate that blood vessel injury, such as that resulting from balloon denudation, upregulates CD44-V6 expression. The upregulation of CD44 on smooth muscle cells may have significant impact on smooth muscle migration and proliferation in the vessel wall.

Uses

Inhibition of CD44 binding

CD44-V6 may be directly involved in smooth muscle cell migration. Thus, CD44-V6, and fragments thereof,can be used as blocking agents to interfere with binding of smooth muscle cells to other cells or to components of the extracellular matrix and thus, migration of smooth muscle cells to sites of vascular injury. CD44 may also act as a mediator of other cellular activities by influencing other adhesion molecules such as integrins.

Compounds can be used inhibit the migration or proliferation of smooth muscle cells by blocking the association of CD44 to a ligand on a second cell or to a component of the extracellular matrix. For example, soluble peptides which bind to CD44 or to the ligand to which CD44 binds may be used to prevent or disrupt CD44 association with a ligand. Peptides, such as a soluble fragment of CD44 extracellular domain or a soluble fragment of a CD44 ligand, could also be used. Such peptides will ordinarily be at least about 10 amino acids, usually about 20 contiguous amino acids, preferably at least 40 contiguous amino acids, more preferably at least 50 contiguous amino acids, and most preferably at least about 60 to 80 or more contiguous amino acids in length. Such peptides can be generated by methods known to those skilled in the art, including proteolytic cleavage of the protein, *de novo* synthesis of the fragment, or genetic engineering, e.g., cloning and expression of a fragment of CD44 cDNA (Aruffo et al., *supra*; Gunthert et al., *supra*; Jackson et al., *supra*; Qi et al., *supra*).

Analogs of the above peptides may also be used to block CD44 binding. Analogs can differ from the native peptides of CD44 by amino acid sequence, or by modifications which do not affect the sequence, or by

both. Modifications (which do not normally alter primary sequence) include *in vivo* or *in vitro* chemical derivitization of polypeptides, e.g., acetylation or carboxylation. Also included are modifications of glycosylation, e.g., those made by modifying the glycosylation patterns of a polypeptide during its synthesis and processing or in further processing steps, e.g., by exposing the polypeptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes.

Soluble compounds, such as portions of known CD44 ligands, e.g., hyaluronic acid, fibronectin, collagen type I and type VI, can also be used to block the association of CD44 with its ligand. Synthetic ligands which bind to CD44, such as those with the binding properties of hyaluronic acid or fibronectin, collagen type I and type VI could also be used. Antibodies or antigen-binding fragments thereof, which are specific for either the extracellular domain of CD44 or the ligand to which CD44 binds, are also useful to block CD44 binding.

To render the therapeutic peptides less susceptible to cleavage by peptidases, the peptide bonds of a peptide may be replaced with an alternative type of covalent bond (a "peptide mimetic"). Where proteolytic degradation of the peptides following injection into the subject is a problem, replacement of a particularly sensitive peptide bond with a noncleavable peptide mimetic will make the resulting peptide more stable and thus, more useful as a therapeutic. Such mimetics, and methods of incorporating them into polypeptides, are well known in the art. Similarly, the replacement of an L-amino acid residue with a D-amino acid is a standard way of rendering the polypeptide less sensitive to proteolysis. Also useful are amino-terminal blocking groups such as t-butyloxycarbonyl, acetyl, theyl,

succinyl, methoxysuccinyl, suberyl, adipyl, azelayl, dansyl, benzyloxycarbonyl, fluorenylmethoxycarbonyl, methoxyazelayl, methoxyadipyl, methoxysuberyl, and 2,4,-dinitrophenyl. Blocking the charged amino- and carboxy-termini of the peptides would have the additional benefit of enhancing passage of the peptide through the hydrophobic cellular membrane and into the cell. This modification may be especially useful in the delivery of peptides into cells, the delivery of fragments of the cytoplasmic domain of CD44 into the cell to inhibit CD44-mediated signal transduction. Liposomal delivery is also favored for intracellular delivery of compounds.

Peptides may be administered to the patient intravenously in a pharmaceutically acceptable carrier such as physiological saline. Such methods are well known to those of ordinary skill in the art. It is expected that an intravenous dosage of approximately 1 to 100 μmoles of the peptide of the invention would be administered per kg of body weight per day. The formulations of this invention are useful for parenteral administration, such as intravenous, subcutaneous, intramuscular, and intraperitoneal.

Other methods of delivery, e.g., diffusion from a stent unpregnated with therapeutic compound, are known in the art.

Inhibition of CD44 expression

The increase of CD44 expression may be an important functional step in smooth muscle cell migration. Thus, manipulation of CD44 expression could be used to prevent or inhibit smooth muscle cell migration. For example, administration of compounds which inhibit CD44 expression would be useful to prevent restenosis after vascular injury.

To decrease expression of CD44 in a smooth muscle cell, an isolated CD44 antisense DNA, may be introduced into target cells of the patient by standard vectors and/or gene delivery systems. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, and adenoviruses, among others. Pharmaceutically acceptable carriers are biologically compatible vehicles which are suitable for administration to an animal: e.g., physiological saline. A therapeutically effective amount is an amount of the nucleic acid of the invention which is capable of producing a medically desirable result in a treated animal, i.e., decreased expression of CD44.

As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Dosages for the compounds of the invention will vary, but a preferred dosage for intravenous administration is from approximately 10^6 to 10^{22} copies of the nucleic acid molecule.

In addition to antisense DNA therapy, compounds which inhibit CD44 expression by interfering with protein synthesis, proper post-translational modification, or translocation and anchoring of the CD44 protein product with respect to the cellular membrane can also be used.

Screening assays

CD44, particularly its V6 isoform, may be considered targets for the development of drugs that it inhibit its action, either in binding to one of its critical ligands or in inhibiting possible intracellular signaling after ligand binding. These drugs would have

application in the prevention of arteriosclerosis, or of post-angioplasty or post-bypass surgery restenosis.

In a screening assay designed to identify compounds which inhibit CD44-mediated smooth muscle cell migration, a sample of cells expressing CD44 on their surface can be incubated in the presence of a candidate compound and a control sample of cells incubated in the absence of the same candidate compound. The migration of each sample of cells can then be compared using methods well known in the art. For example, movement of cells can be evaluated using a Boyden chamber or any multiwell chamber in which the wells of the chamber are separated by a membrane through which cells are capable of passing, e.g., a polycarbonate membrane with pores large enough (e.g, 3 μm) to accommodate the size of migrating cells. Treated or untreated cells can be added to one well of the multiwell chamber and movement of cells from one well to the other well of the chamber measured. A decrease in the amount of migration of cells from one well to the other well in the presence of candidate compound compared to that in the absence of candidate compound indicates that the compound inhibits CD44-mediated smooth muscle cell migration.

In a screening assay designed to identify compounds which inhibit proliferation of smooth muscle cells, a sample of cells expressing CD44 on the surface can be incubated in the presence of a candidate compound. Similarly, a control sample of cells is incubated in the absence of the same candidate compound. Following incubation, proliferation of each sample of cells can be evaluated by exposing the cells to medium containing [^{3}H]-thymidine. Incorporation of thymidine into the DNA of the cells indicates that the cells are replicating or proliferating. The amount of [^{3}H]-thymidine incorporated into each sample of cells is can be measured and

corrected with cell proliferation. A decrease in the amount of incorporated [^{3}H]-thymidine in cells that were contacted with candidate compound compared to the amount in cells that were not contacted with the compound indicates that the candidate compound inhibits smooth muscle cell proliferation.

A screening assay to identify compounds which are capable of inhibiting expression of CD44 in smooth muscle cells can be carried out as follows. A sample of activated smooth muscle cells, e.g. cultured smooth muscle cells, can be incubated in the presence of a candidate compound. Similarly, a sample of control cells can be incubated in the absence of the compound. Each sample of cells can then be evaluated for the expression of CD44. For example, each sample of cells can be incubated with a CD44-specific antibody and the cells evaluated for binding of the antibody by immunofluorecent staining or FACS. The amount of antibody binding is correlated with the level of expression of CD44. CD44 expression can also be measured at the level of gene transcription. For example, CD44 transcripts can be measured by Northern blotting techniques using CD44-specific DNA probes or by PCR using CD44-specific DNA primers. A decrease in the amount CD44 expression in treated cells, as measured by any such technique compared, compared to the amount in untreated cells indicates that the candidate compound is capable of inhibiting the expression of CD44 in smooth muscle cells.

EXAMPLE 1: Demonstration of CD44 and CD44-V6 on vascular smooth muscle cells *in vivo* and *in vitro*

Identification of CD44 and CD44-V6 on cultured rat aortic smooth muscle cells (10% FCS) was established by flow cytometry analysis using monoclonal antibodies and FITC labeled secondary antibody. As seen in Figs. 6A-6D,

the standard form of CD44 was expressed at levels considerably higher than β_1 or β_3 integrins. CD44-V6 was expressed at levels similar to those of β_1 and β_3 integrins but lower than standard CD44. Expression of CD44 and its variant were lower in quiescent (0.4% FCS) versus activated cells (10% FCS).

Vascular smooth muscle cells were analyzed to determine the level of CD44 and CD44-V6 expression *in vivo*. The effect of cellular stimulation on expression of these proteins was also determined. One method of stimulating vascular smooth muscle cells is through arterial wall injury. Carotid artery specimens after injury were compared to control (uninjured) vessels by immunocytochemical staining. CD44 and CD44-V6 levels were found to be markedly upregulated after injury. Thus, vascular smooth muscle cells express both CD44 and CD44-V6 *in vitro* and *in vivo*, and levels are upregulated after serum or mechanical stimulation.

EXAMPLE 2: Determination of mRNA levels *in vitro* and *in vivo*

To determine if the increase in expression of CD44 and CD44-V6 was regulated at the transcriptional level, Northern blot analysis was done. Fig. 7 shows a comparison of mRNA expression of CD44 in quiescent (0.4% FCS) to that in activated (10% FCS) smooth muscle cells. Three bands were detected in each lane corresponding to sizes of known CD44 transcripts. However, no significant difference was seen between quiescent and activated vascular smooth muscle cells in culture. Since passaged RASMC represent a synthetic, dedifferentiated phenotype it is possible that these cells, despite being cultured in 0.4% FCS, are not truly quiescent. Thus, mRNA levels of cells grown on matrigel were also evaluated. Matrigel is a solubilized basement membrane matrix which has

recently been used to maintain cultured cells in a highly differentiated phenotype (Li et al., 1994, J. Biol. Chem., 269:19653-19658). Figs. 8A and 8B show a comparison of CD44 expression in cells grown on plastic in 10% FCS (growing and confluent) to cells grown on matrigel. A decrease in mRNA expression was seen in cells grown on matrigel.

To assess whether differences in mRNA expression can be detected *in vivo*, the rat carotid artery injury model was used. Rats at 2 and 5 days after carotid injury were compared to control (uninjured) rats. A 3.6-fold increase in CD44 expression was detected at 2 days after injury (Figs. 10A and 10B). A 7.5-fold increase was seen in CD44-V6 expression at this same time point (Figs 9A and 9B). In both cases mRNA levels decreased by 5 days although they did not return to the baseline level. These data suggest that the changes seen in CD44 and CD44-V6 expression at the protein level are due to changes at the transcriptional level.

EXAMPLE 3: *In situ* Hybridization analysis

In order to confirm that the specific cell populations expressing CD44 and CD44-V6 are smooth muscle cells, *in situ* analysis was performed on rat carotid artery injury specimens using digoxigenin labeled riboprobes. Smooth muscle cells in the media and neointima were found to have considerably more labeling than the control. These data confirm that the upregulation of CD44 and CD44-V6 mRNA levels occurs in the vascular smooth muscle cells.

EXAMPLE 4: Augmentation of [^{3}H]-thymidine incorporation by hyaluronate and anti-CD44 antibodies

Using the natural ligand for CD44, hyaluronate, as well as anti-CD44 monoclonal antibodies, the

incorporation of [^{3}H]-thymidine by vascular smooth muscle cells in culture was evaluated. As shown in Figs. 11A and 11B, hyaluronate at a concentration of 1 mg/ml resulted in a two-fold increase in [^{3}H]-thymidine incorporation in both rat and human vascular smooth muscle cells. The increase observed was similar to that seen with 5% fetal calf serum, a powerful stimulant. A steady decline in [^{3}H]-thymidine incorporation was noted at progressively lower doses.

Two different monoclonal antibodies to rat CD44 induced in a two-fold increase in [^{3}H]-thymidine incorporation in RASMC (Fig. 12B). Similarly, incubation of HASMC with a monoclonal antibody to human CD44 also resulted in a two-fold increase in proliferation (Fig. 12A). These results, using two different ligands to CD44 (hyaluronate and monoclonal antibodies) in two different tissue types strongly suggest that CD44 plays an important role in vascular smooth muscle activation.

Other embodiments are within the claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: HE, Qi
HABER, Edgar

(ii) TITLE OF INVENTION: CD44 EXPRESSION IN SMOOTH MUSCLE CELLS

(iii) NUMBER OF SEQUENCES: 4

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 08/183,156
(B) FILING DATE: 18 January 1994
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:

(viii) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: 00246/182001

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(C) TELEX: 200154

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	20
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGCCAGTGAC AGGTTCCATT 20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	20
(B) TYPE:	nucleic acid
(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

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

TGTTGTGTCT TTTCAAGTTA 20

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

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(B) TYPE:	nucleic acid
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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:	25
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(C) STRANDEDNESS:	single
(D) TOPOLOGY:	linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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What is claimed is:

1. A method of treating arteriosclerosis or vascular restenosis in an animal comprising inhibiting the expression of CD44 on smooth muscle cells in said animal.

2. The method of claim 1, wherein said expression is inhibited by introducing CD44 antisense DNA into said smooth muscle cells.

3. The method of claim 1, wherein the isoform of said CD44 is V6.

4. The method of claim 1, wherein expression of CD44 in said smooth muscle cell is upregulated as a result of blood vessel injury.

5. A method of decreasing migration of a smooth muscle cell comprising inhibiting the expression of CD44 in said smooth muscle cell.

6. The method of claim 5, wherein said expression is inhibited by introducing CD44 antisense DNA into said smooth muscle cells.

7. The method of claim 5, wherein the isoform of said CD44 is V6.

8. The method of claim 5, wherein expression of CD44 in said smooth muscle cell is upregulated as a result of blood vessel injury.

9. A method of decreasing proliferation of a smooth muscle cell comprising inhibiting the expression of CD44 in said smooth muscle cell.

10. A method of decreasing migration of a smooth muscle cell, comprising contacting said smooth muscle cell with a compound which blocks the binding of said smooth muscle cell to a component of the extracellular matrix.

11. The method of claim 10, wherein said compound is a CD44-specific antibody or an antigen-binding fragment thereof.

12. The method of claim 11, wherein said antibody or antigen-binding fragment thereof binds to the V6 domain of CD44.

13. The method of claim 10, wherein said compound is a soluble fragment of CD44.

14. The method of claim 10, wherein said compound is a soluble fragment of CD44 comprising the V6 domain of said CD44.

15.The method of claim 10, wherein said compound binds to the extracellular domain of CD44.

16. The method of claim 15, wherein said compound has the binding properties of hyaluronic acid or fibronectin.

17. The method of claim 10, wherein said compound inhibits CD44-mediated signal transduction.

18. The method of claim 17, wherein said compound is a soluble fragment of the cytoplasmic domain of CD44.

19. A method of decreasing migration of a smooth muscle cell, comprising contacting said smooth muscle cell with a compound which blocks the binding of said smooth muscle cell to a second cell.

20. A method of decreasing proliferation of a smooth muscle cell, comprising contacting said smooth muscle cell with a compound which blocks the binding of said smooth muscle cell to a component of the extracellular matrix.

21. A method of decreasing proliferation of a smooth muscle cell, comprising contacting said smooth muscle cell with a compound which inhibits CD44-mediated signal transduction.

22. The method of claim 21, wherein said compound is a soluble fragment of the cytoplasmic domain of CD44.

23. A method of decreasing proliferation of a smooth muscle cell, comprising contacting said smooth muscle cell with a compound which blocks the binding of said smooth muscle cell to a second cell.

24. A method of screening candidate compounds to identify a compound capable of inhibiting CD44-mediated smooth muscle cell migration, comprising

(a) providing smooth muscle cell bearing CD44 on its surface;

(b) contacting said smooth muscle cell with a candidate compound;

(c) determining the amount of migration of said smooth muscle cell, wherein a decrease in the presence of said candidate compound compared to the amount in the absence of said candidate compound indicates that said candidate compound inhibits CD44-mediated smooth muscle cell migration.

25. A method of screening candidate compounds to identify a compound capable of inhibiting CD44-mediated smooth muscle cell proliferation, comprising

(a) providing smooth muscle cell bearing CD44 on its surface;

(b) contacting said smooth muscle cell with a candidate compound;

(c) determining the amount of proliferation of said smooth muscle cell, wherein a decrease in the presence of said candidate compound compared to the amount in the absence of said candidate compound indicates that said candidate compound inhibits CD44-mediated smooth muscle cell proliferation.

26. A method of screening candidate compounds to identify a compound capable of inhibiting expression of CD44 in smooth muscle cells, comprising

(a) providing an activated smooth muscle cell bearing CD44 on its surface;

(b) contacting said smooth muscle cell with a candidate compound;

(c) determining the amount of CD44 expression of said smooth muscle cell, wherein a decrease in the presence of said candidate compound compared to the amount in the absence of said candidate compound indicates that said candidate compound inhibits CD44-expression in smooth muscle cells.

A v6 Variant
on Rat Smooth Muscle Cells

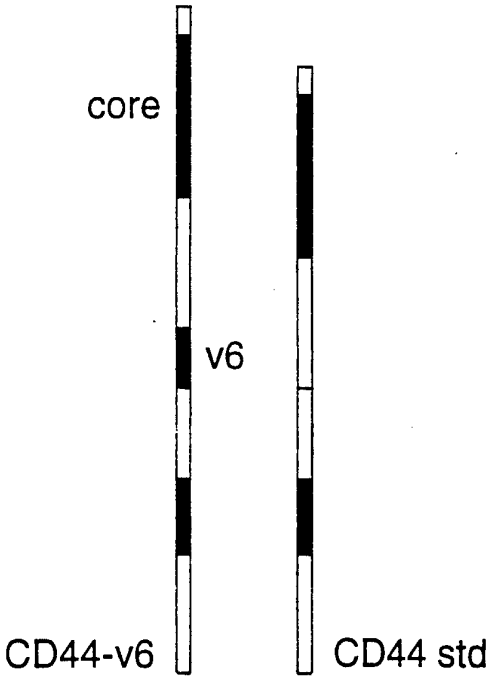


FIG. 1

Proteoglycan CD44

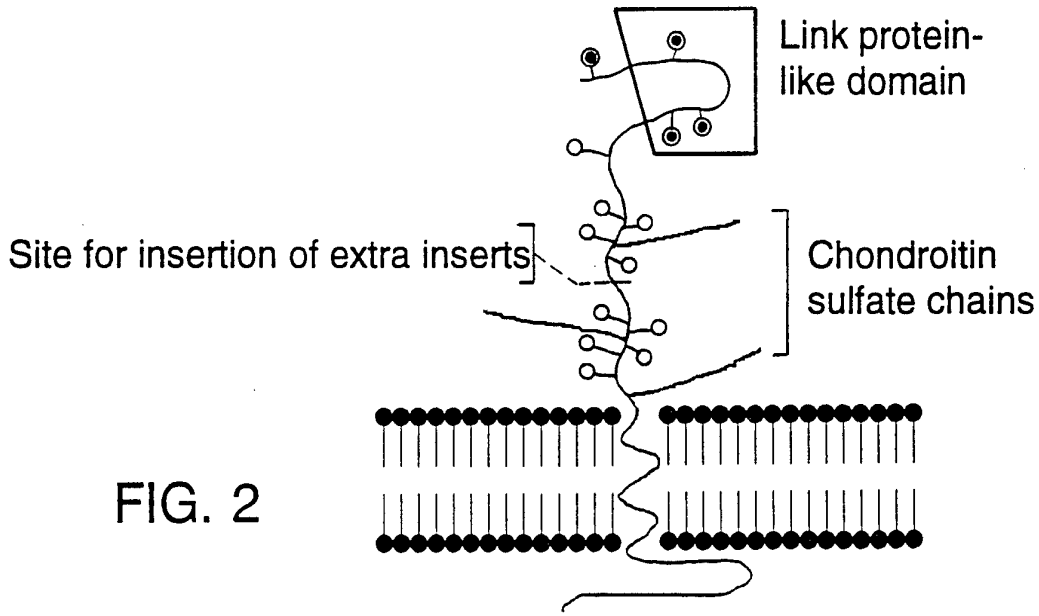


FIG. 2

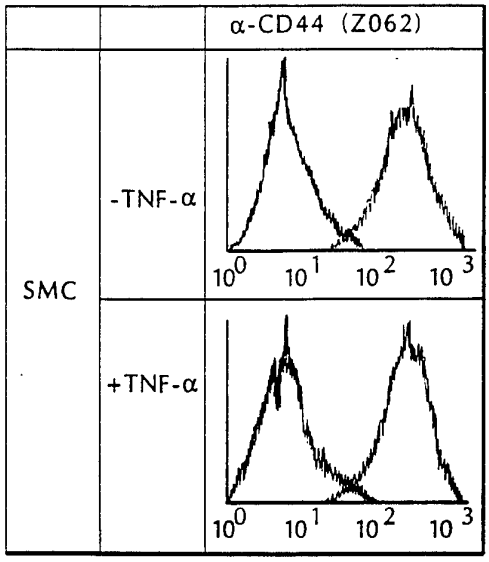


FIG. 3

v6 Variants on Rat Smooth Muscle Cells

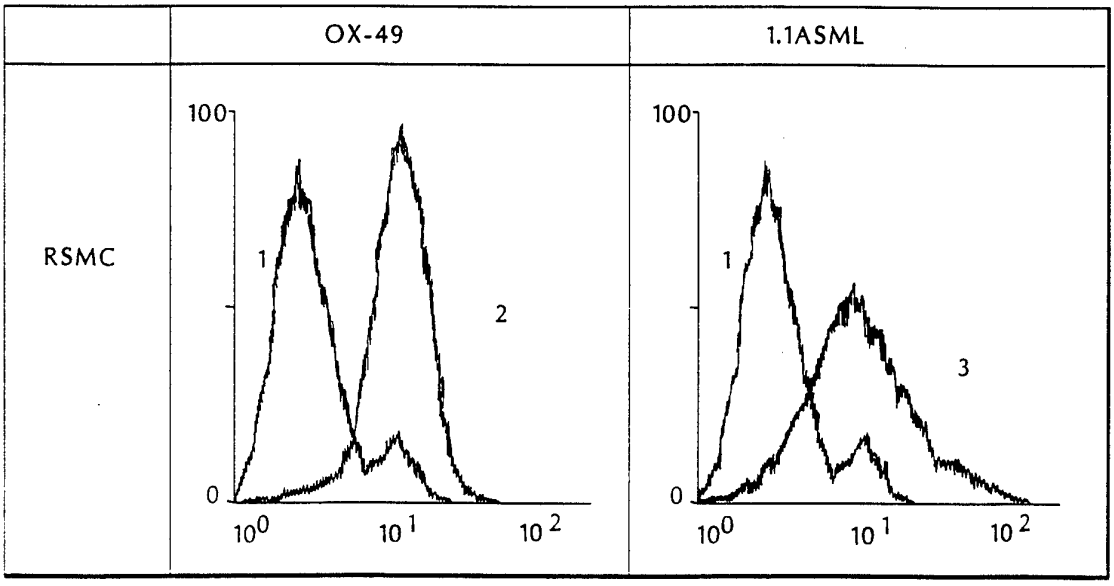


FIG. 4

FACS Analysis of Surface Antigens

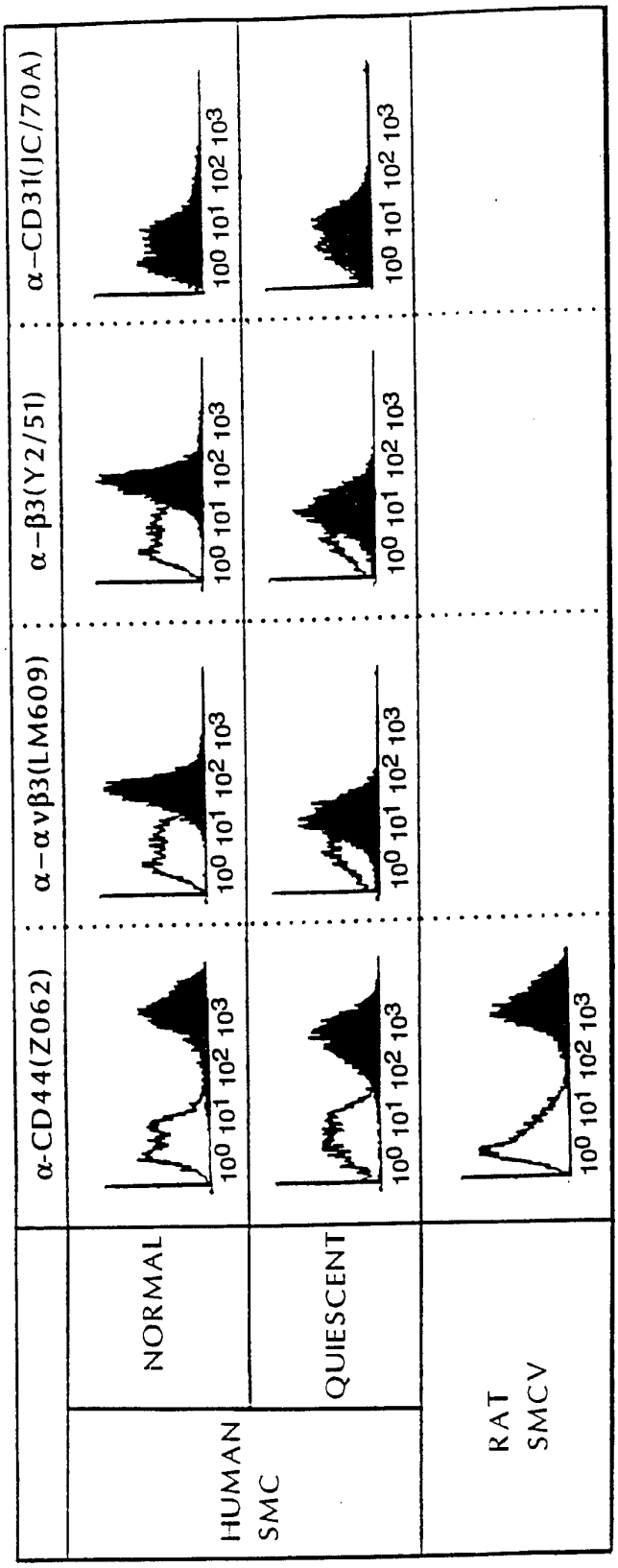


FIG. 5

FIG. 6

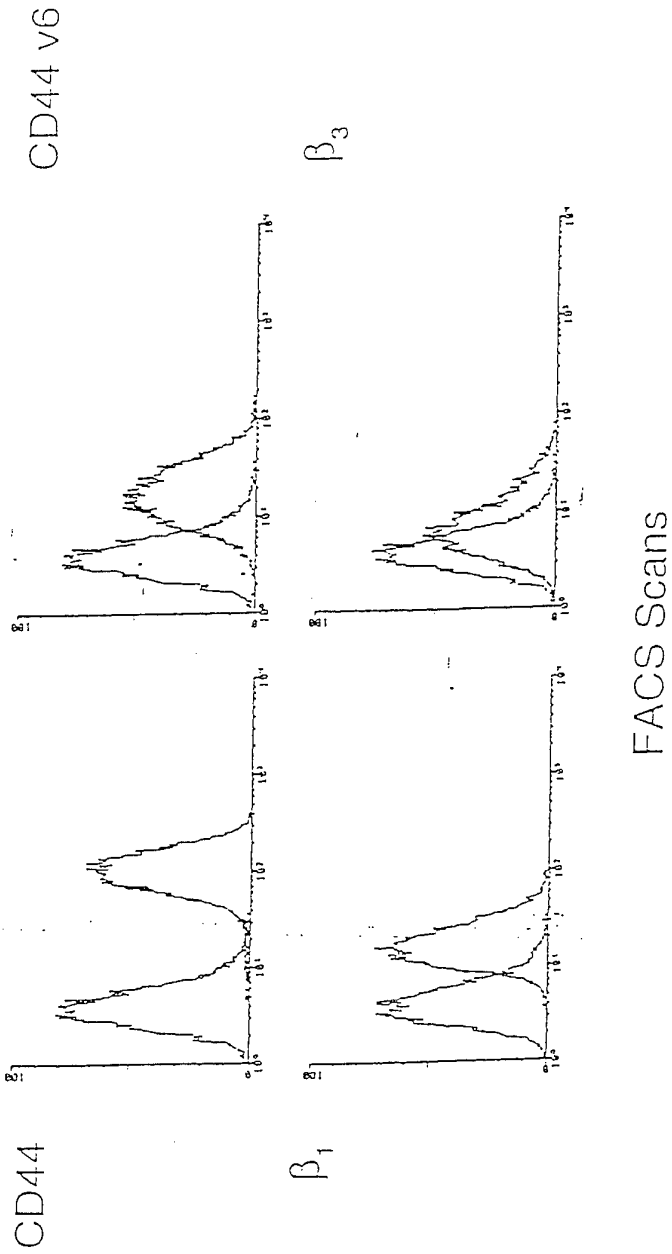


FIG. 7

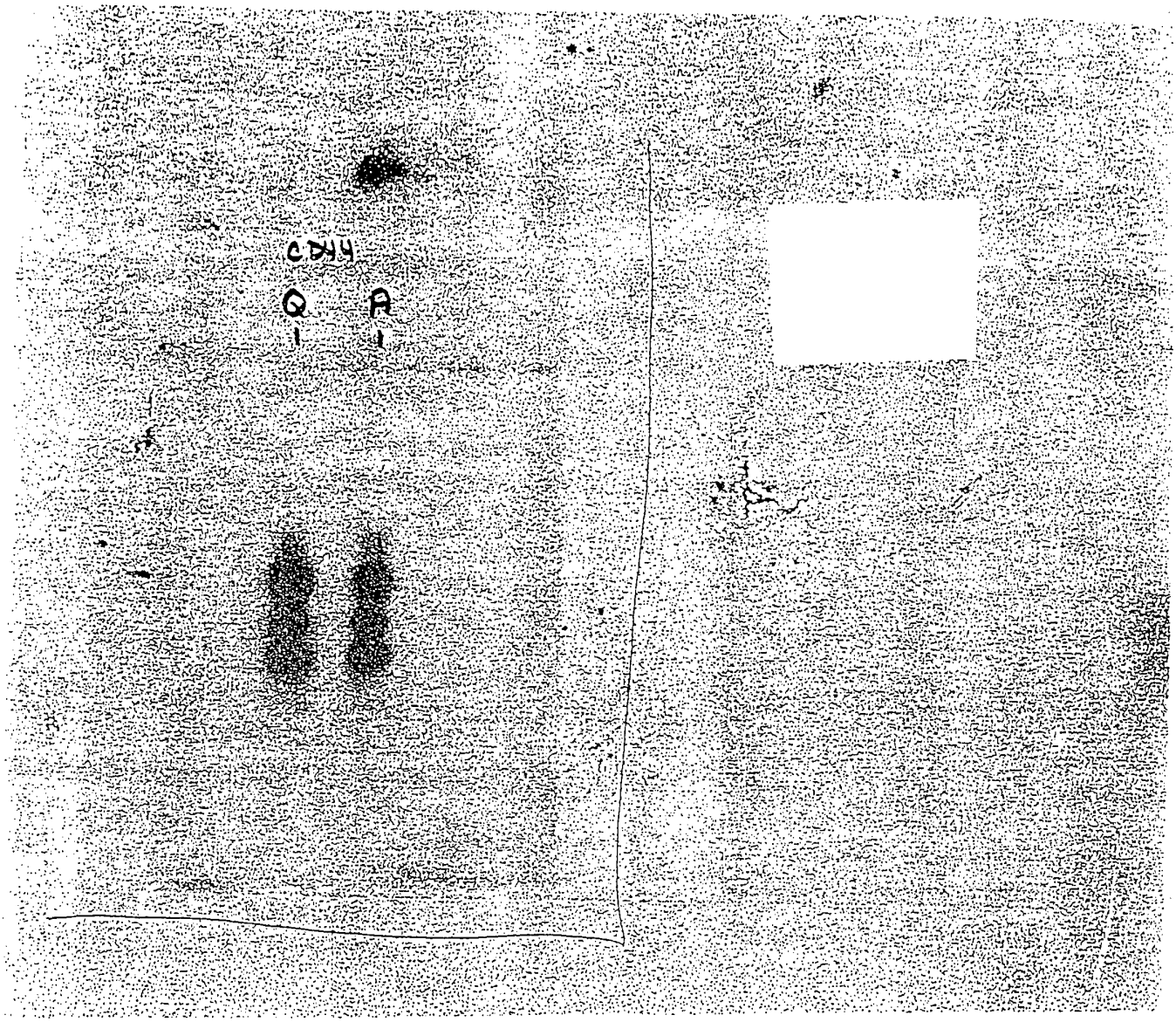


FIG. 8A

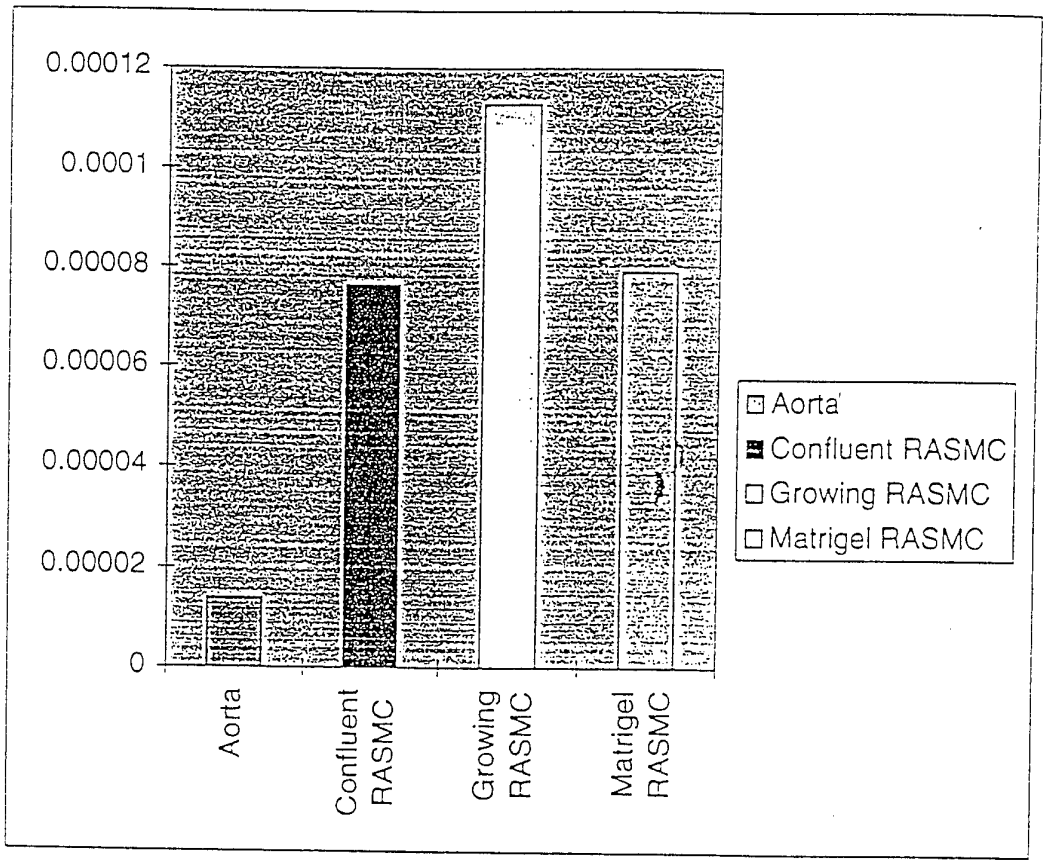
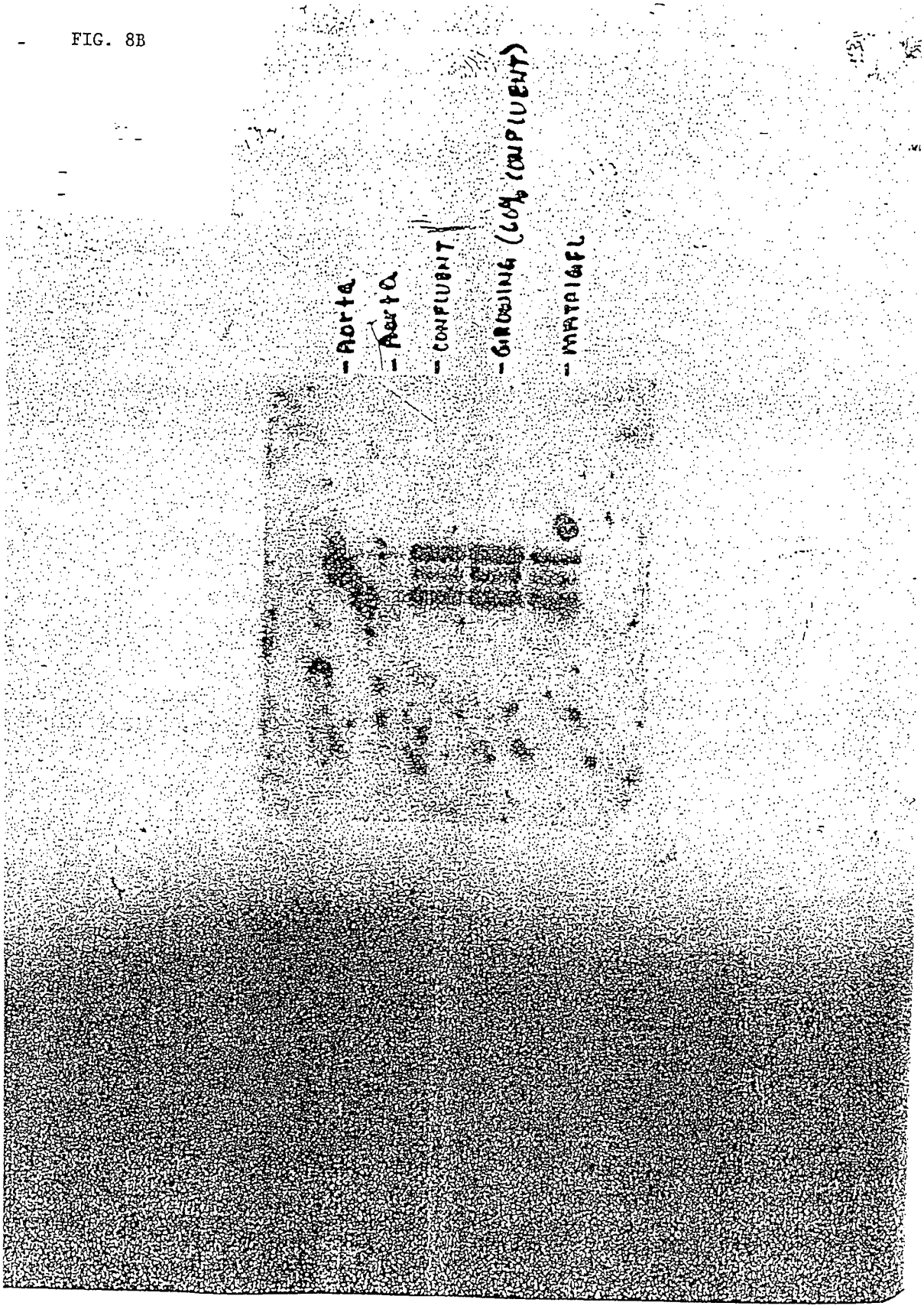

0.00012
0.0001
0.00008
0.00006
0.00004
0.00002
0
Aorta
Confluent RASMC
Growing RASMC
Matrigel RASMC
Aorta
Confluent RASMC
Growing RASMC
Matrigel RASMC

FIG. 8B



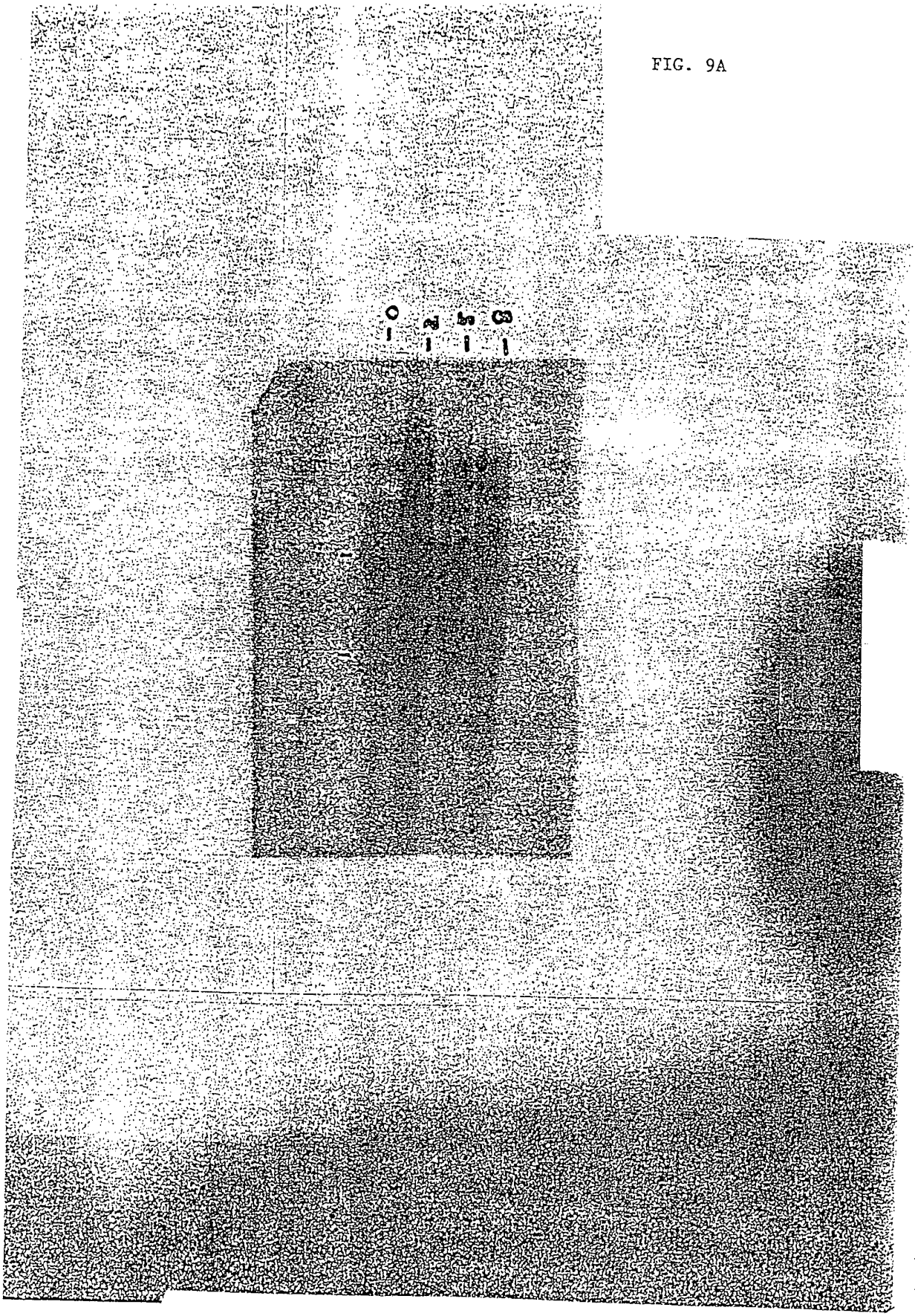
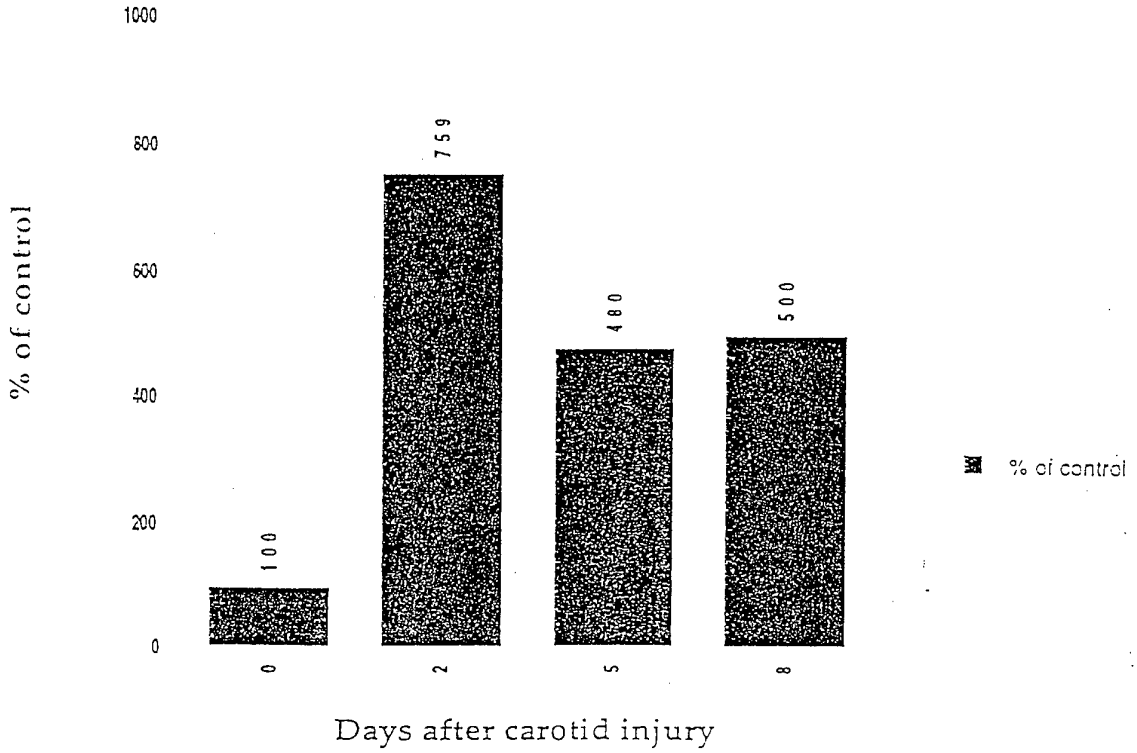


FIG. 9A

FIG. 9B


1000
800
600
400
200
0
% of control
100
759
480
500
0
2
5
8
Days after carotid injury
% of control

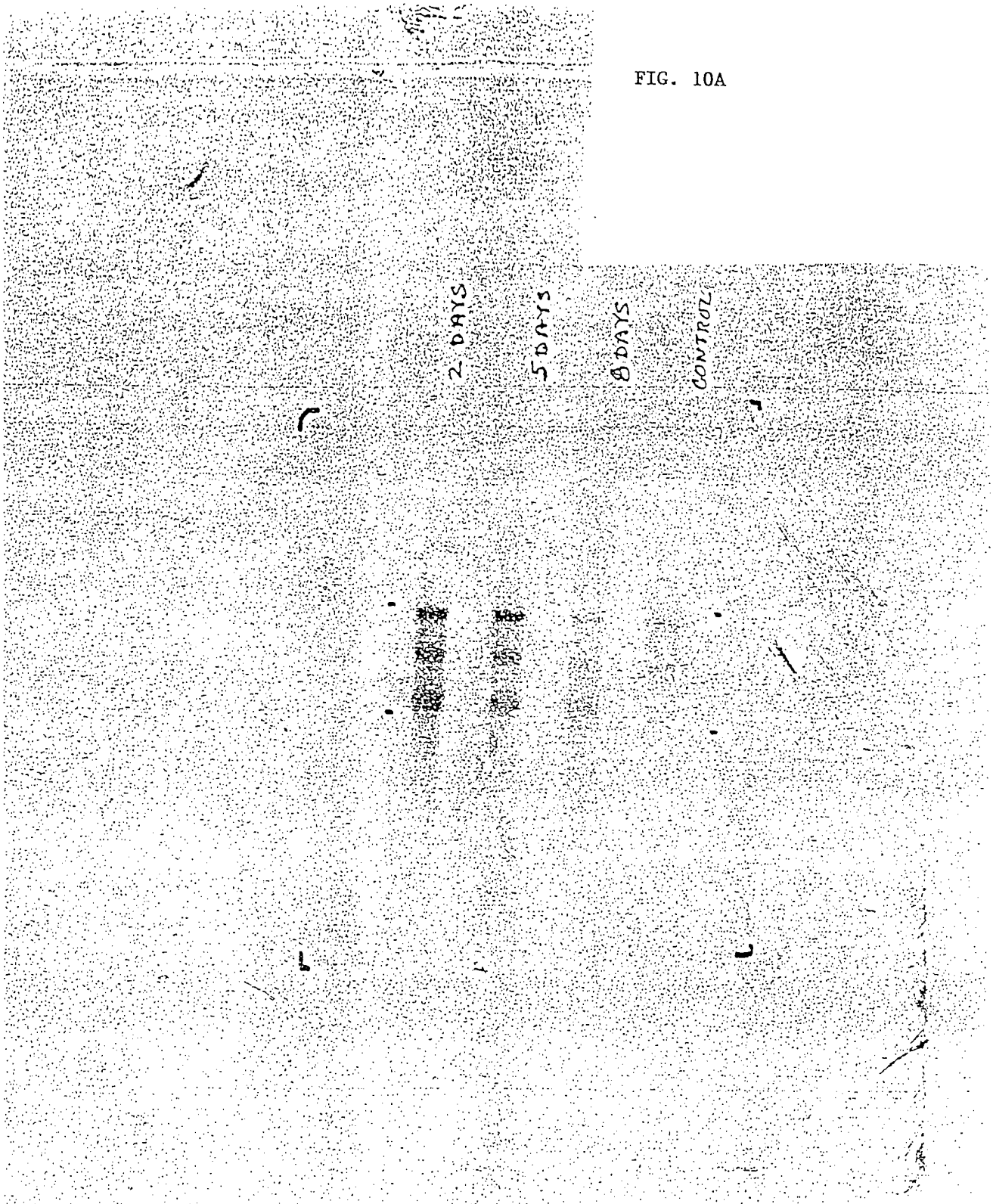


FIG. 10A

FIG. 10B

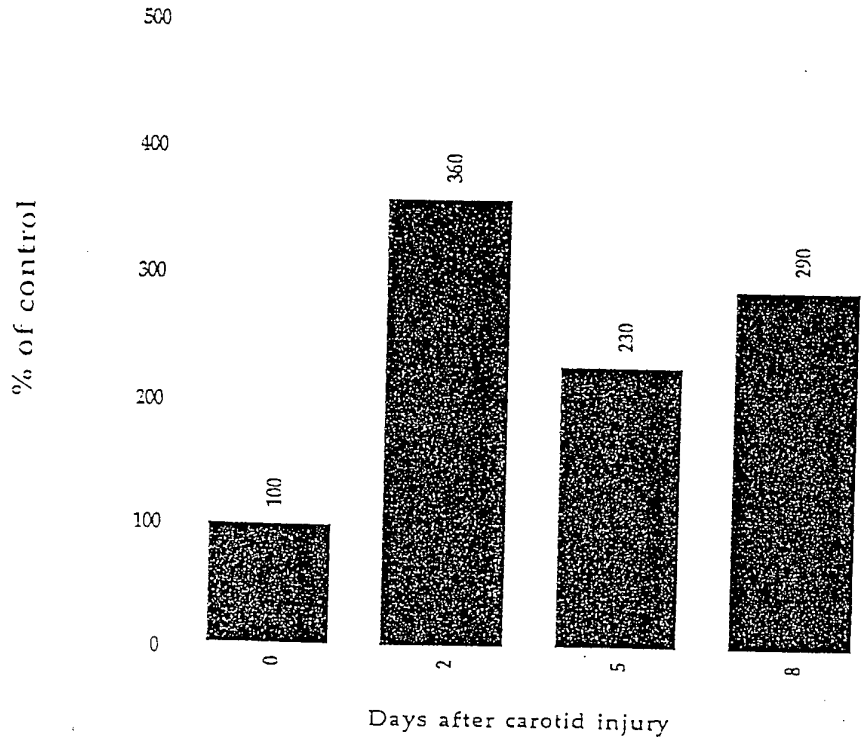

500
400
300
200
100
0
% of control
100
360
230
290
0
2
5
8
Days after carotid injury

FIG. 11A

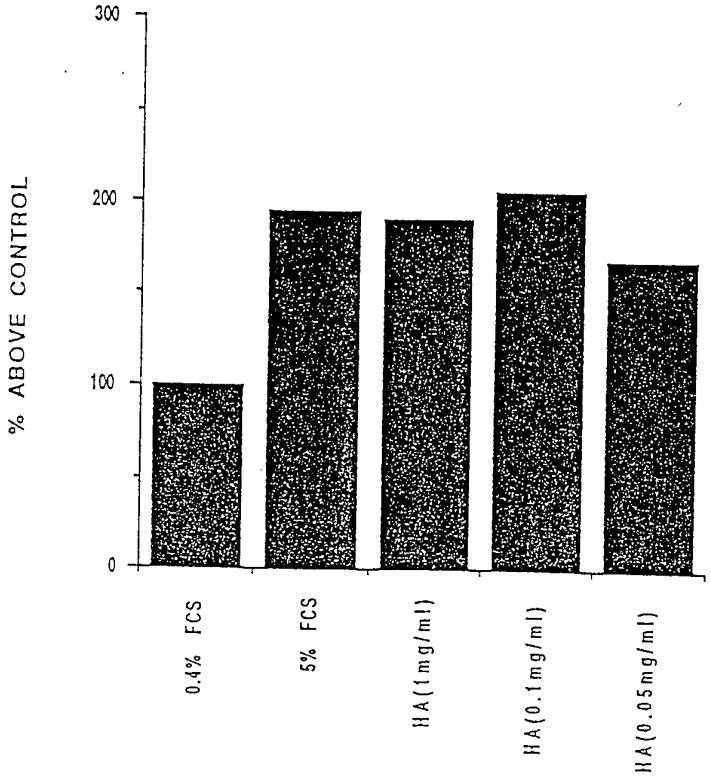

% ABOVE CONTROL
300
200
100
0
0.4% FCS
5% FCS
HA(1mg/ml)
HA(0.1mg/ml)
HA(0.05mg/ml)

FIG. 11B

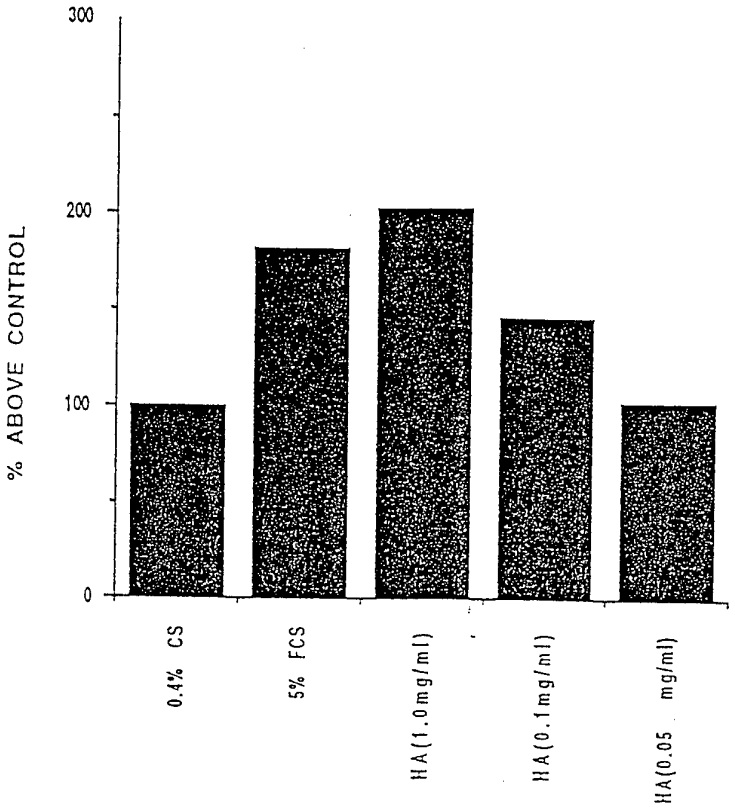

300
200
100
0
% ABOVE CONTROL
0.4% CS
5% FCS
HA(1.0mg/ml)
HA(0.1mg/ml)
HA(0.05 mg/ml)

FIG. 12A

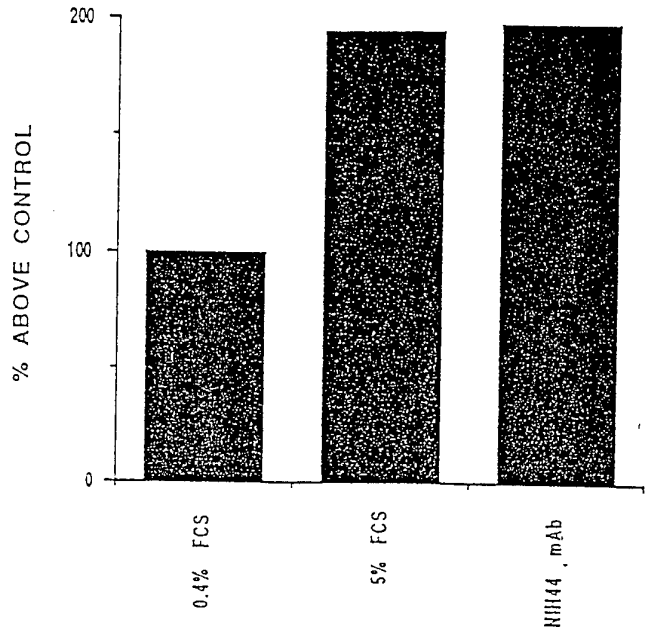
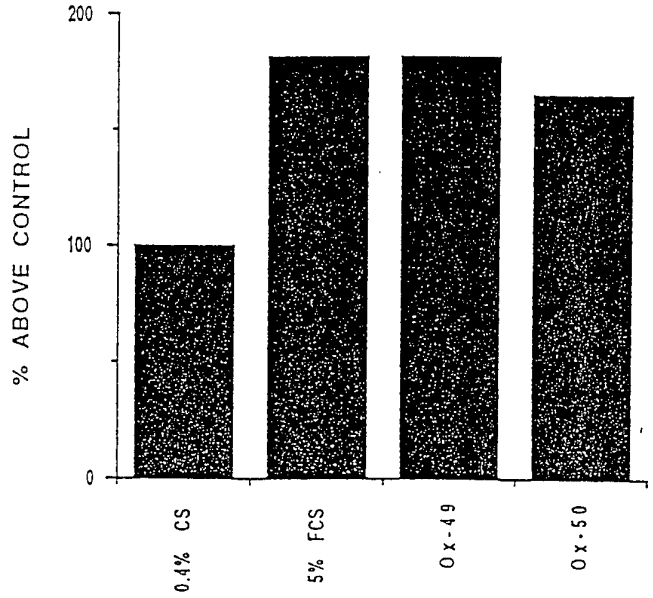


FIG. 12B



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00740

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : A61K 48/00; G01N 33/50

US CL :514/44; 435/7.2

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)

U.S. : 514/44; 435/7.2

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

None

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, Dialog,
search terms: CD44, arteriosclerosis, smooth muscle cells, isoform

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Journal of Biological Chemistry, Volume 267, No. 7, issued 05 March 1992, Jackson et al., "Multiple variants of the human lymphocyte homing receptor CD44 generated by insertions at a single site in the extracellular domain", pages 4732-4739, see entire reference.	1-23
Y	Cell, Volume 65, issued 05 April 1991, Gunthert et al., "A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells", pages 13-24, see entire reference.	1-23

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

27 APRIL 1995

Date of mailing of the international search report

01 MAY 1995

Name and mailing address of the ISA/US
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00740

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Science, Volume 257, issued 31 July 1992, Arch et al., "Participation in normal immune responses of a metastasis-inducing splice variant of CD44", pages 682-685, see entire article.	3, 7, 10, 11, 12, 19
Y	Journal of Cell Biology, issued December 1992, Volume 119, No. 6., He et al., "Molecular isoforms of murine CD44 and evidence that the membrane proximal domain is not critical for hyaluronate recognition", pages 1711-1719, see entire reference.	13, 14, 22
Y	Cell, Volume 61, issued 29 June 1990, Aruffo et al., "CD44 is the principal cell surface receptor for hyaluronate", pages 1303-1313, see entire article.	13, 14, 22
Y	Anti-Cancer Drug Design, Volume 6, issued December 1991, Mirabelli et al., "In vitro and in vivo pharmacologic activities of antisense oligonucleotides", pages 647-661, see entire reference.	1-9, 24-26