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(54) Title: METHOD OF DIAGNOSING, PREDICTING AND MONITORING THE PROGRESS OF AN INFLAMMATORY RESPONSE CHARACTERISED BY ANALYSIS OF AN ACTIVIN PROTEIN COMPRISING A β_B SUBUNIT

(57) Abstract: The present invention relates generally to a method of diagnosing, predicting and/or monitoring the development or progress of an inflammatory response in a mammal. More particularly, the present invention relates to a method of diagnosing, predicting and/or monitoring the development or progress of an inflammatory response by analysis of activin β_B expression levels either in a subject mammal or in a biological sample derived from said mammal. The present invention further provides a method for predicting, diagnosing and/or monitoring conditions associated with or characterised by the onset of an inflammatory response. Also provided are diagnostic agents useful for detecting activin β_B expression levels.

Method of diagnosing, predicting and monitoring the progress of an inflammatory response characterised by analysis of an activin protein comprising a β_B subunit

FIELD OF THE INVENTION

The present invention relates generally to a method of diagnosing, predicting and/or monitoring the development or progress of an inflammatory response in a mammal. More particularly, the present invention relates to a method of diagnosing, predicting and/or monitoring the development or progress of an inflammatory response by analysis of activin β_B expression levels either in a subject mammal or in a biological sample derived from said mammal. The present invention further provides a method for predicting, diagnosing and/or monitoring conditions associated with or characterised by the onset of an inflammatory response. Also provided are diagnostic agents useful for detecting activin β_B expression levels.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications referred to by author in this specification are collected alphabetically at the end of the description.

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in Australia.

Mammals are required to defend themselves against a multitude of pathogens including viruses, bacteria, fungi and parasites, as well as non-pathogenic insults such as tumours and toxic, or otherwise harmful, agents. In response, effector mechanisms have evolved which are capable of mounting a defence against such antigens. These mechanisms are mediated by soluble molecules and/or by cells.

In the context of these effector mechanisms, inflammation is a complex multifaceted response to disease or injury which is regulated by the release of a cascade of cytokines.

These cytokines are classified in general terms as pro- or anti-inflammatory cytokines and the critical balance between release and activity of cytokines with opposing actions regulates the inflammatory response to prevent it from becoming overt or understated. If the inflammatory response continues unchecked and is overt then the host may suffer associated tissue damage. Conversely, a poor or understated inflammatory response may mean uncontrolled infection resulting in chronic illness and host damage. Regulation of the inflammatory response is important at both the systemic level and the local level.

The discovery of the detailed processes of inflammation has revealed a close relationship between inflammation and the immune response. There are five basic indicators of inflammation, these being redness (rubor), swelling (tumour), heat (calor), pain (dolor) and deranged function (functio laesa). These indicators occur due to extravasation of plasma and infiltration of leukocytes into the site of inflammation. Consistent with these indicators, the main characteristics of the inflammatory response are therefore:

(i) vasodilation – widening of the blood vessels to increase the blood flow to the infected area;

(ii) increased vascular permeability – this allows diffusible components to enter the site;

(iii) cellular infiltration – this being the directed movement of inflammatory cells through the walls of blood vessels into the site of injury;

(iv) changes in biosynthetic, metabolic and catabolic profiles of many organs; and

(v) activation of cells of the immune system as well as of complex enzymatic systems of blood plasma.

The degree to which these characteristics occur is generally proportional to the severity of the injury and/or the extent of infection.

The inflammatory response can be broadly categorised into several phases. The earliest, gross event of an inflammatory response is temporary vasoconstriction, i.e. narrowing of blood vessels caused by contraction of smooth muscle in the vessel walls, which can be seen as blanching (whitening) of the skin. This is followed by several phases that occur over minutes, hours and days later, as follows:

(i) The acute vascular response follows within seconds of a tissue insult and lasts for some minutes. It is characterised by vasodilation and increased capillary permeability due to alterations in the vascular endothelium, leading to increased blood flow (hyperaemia) that causes redness (erythema) and the entry of fluid into the tissues (oedema).

(ii) If there has been sufficient damage to the tissues, or if infection has occurred, the acute cellular response takes place over the next few hours. The hallmark of this phase is the appearance of granulocytes, particularly neutrophils, in the tissue. These cells first attach themselves to the endothelial cells within the blood vessels (margination) and then cross into the surrounding tissue (diapedesis). If the vessel is damaged, fibrinogen and fibronectin are deposited at the site of injury, platelets aggregate and become activated and clot formation occurs.

(iii) If damage is sufficiently severe, a chronic cellular response may follow over the next few days. A characteristic of this phase of inflammation is the appearance of a mononuclear cell infiltrate composed of macrophages and lymphocytes. The macrophages are involved in microbial killing, in clearing up cellular and tissue debris, and are also thought to play a significant role in remodelling tissue.

(iv) Over the next few weeks, resolution may occur wherein normal tissue architecture is restored. Blood clots are removed by fibrinolysis. If it is not possible to return the tissue to its original form, scarring may occur from in-filling with fibroblasts, collagen, and new endothelial cells. Generally, by this time any infection will have

been overcome, although this is not always the case and may result in further immunological responses, such as granuloma formation.

Inflammation is often considered in terms of acute inflammation that includes all the events of the acute vascular and acute cellular response (1 and 2 above), and chronic inflammation that includes the events during the chronic cellular response and resolution or scarring (3 and 4).

It should be understood, however, that in addition to the occurrence of inflammatory responses in a localised fashion in tissue which is damaged, infected or subject to an autoimmune response, for example, inflammatory responses may also occur systemically, such as in the case with sepsis.

In relation to sepsis, in particular, this condition is a major cause of morbidity and mortality worldwide and is the leading non-coronary cause of death in intensive care units. More than 700,000 cases of severe sepsis occur in the US annually at a healthcare cost of $17 billion annually.

Intense interest has focussed on the ability to discriminate between those patients who will die from sepsis and those who will survive. A number of diagnostic tests including body temperature, leukocyte count and various blood markers such as C-reactive protein, procalcitonin and various cytokines have been evaluated. While a number of these show predictive value in discriminating patient outcome, there is a need to continue to evaluate new markers or combinations of markers to improve diagnostic accuracy.

Accordingly, in light of the wide-ranging impact of inflammatory responses, there is an ongoing need to elucidate the complex mechanisms by which it functions. By identifying these mechanisms there is thereby provided scope for developing means of appropriately diagnosing, monitoring and/or treating inflammatory responses.

Inhibin, activin, and follistatin are three families of polypeptides originally isolated and characterized from ovarian follicular fluid based on their modulation of follicle stimulating hormone release from pituitary cell culture. In addition to their effects on follicle stimulating hormone synthesis and secretion, inhibin and activin have other biological functions. By contrast, the physiological significance of follistatin was obscure, until it was discovered that follistatin is a binding protein to activin.

Activins, composed of two β-subunits, β_A, β_B, β_C, β_D, and/or β_E are members of the transforming growth factor (TGF)-β superfamily [Vale *et al.*, 1990, Handbook of Experimental Physiology, Vol. 95, Eds. Sporn & Roberts, Springer-Verlag, Berlin pp211-248]. Multimeric protein forms of activin include the homodimeric forms (Activin A - $\beta_A\beta_A$, Activin B - $\beta_B\beta_B$, Activin C - $\beta_C\beta_C$, Activin D - $\beta_D\beta_D$, and Activin E - $\beta_E\beta_E$) and the heterodimeric forms (for example, Activin AB - $\beta_A\beta_B$, Activin AC - $\beta_A\beta_C$, Activin AD - $\beta_A\beta_D$, or Activin AE - $\beta_A\beta_E$). The activins are multifunctional proteins. For example, Activin A, although originally identified as a regulator of follicle stimulating hormone release, is now known to exhibit the pleiotropic range of functional activities which are characteristic of most cytokines. Activins, like their related proteins, inhibins (which consist of a dimer of a structurally related but dissimilar α subunit and an activin β subunit) can bind to activin type II receptors. However, only activins are able to recruit type I receptors to form an active complex, triggering intracellular Smad signalling pathways and thereby influencing cellular function at the transcriptional level. At present, activin A, AB and B have been shown to demonstrate typical receptor-mediated agonist activity. Activin B has been reported to display less biological activity than activin A [Nakamura *et al.*, *Journal of Biological Chemistry,* 267, 16385-16389, 1992]. This may be associated with variation in the availability of specific type I receptors, differentially recruited by activin A and B [Tsuchida *et al.*, 2004 *Molecular and Cellular Endocrinology* 220, 50-65].

Follistatin functions as a biological regulator of activin. In fact, it was originally identified as an activin-binding protein. Follistatin is a monomeric protein which binds to activin with high affinity and is believed to thereafter lead to lysosomal degradation of the complexed activin. Follistatin comprises a number of post-translational and glycosylation

variants. However, the two major isoforms are the full length follistatin 315, which is believed to be the predominant circulating isoform, and the 288 isoform, which has a strong affinity for heparin sulphate proteoglycans and is largely a cell membrane-associated isoform (Phillips and deKretser, 1998, *Frontiers in Neuroendocrinology* 19:287-322).

Activin affects the growth and differentiation of many cell types, stimulates the secretion of follicle-stimulating hormone from the pituitary gland and inhibits growth hormone, prolactin, and adrenocorticotropin release [Billestrup *et al.*, *Molecular Endocrinology* 1990 4:356–362; Kitaoka *et al.*, *Biochemical and Biophysical Research Communications* 1988 157:48–54; Vale *et al.*, *Nature* 1986, 321:776–779]. Activin A was first characterized for its ability to stimulate follicle stimulating hormone (FSH) from the pituitary, a capacity shared by activin B [Nakamura *et al.*, 1992, *supra;* Van Dijk *et al.*, 1995, *Annals of the New York Academy of Science* 762, 319-330]. However, activin A is now known to have many more properties besides this initial function for which it was first isolated. Both activin A and B participate in foetal development, with their respective mouse knockouts presenting distinct phenotypic anomalies. Knockouts of activin A exhibit neonatal lethal phenotypic defects [Vassalli *et al.*, 1994, *Genes and Development*, 8:414-427; Matzuk *et al.*, 1995, *Nature* 374: 354-356] but substitution of the β_A gene with β_B provides partial rescue of this phenotype [Brown *et al.*, 2000, *Nature Genetics,* 25:453-457], suggesting some overlap in the activities of activin A and B. In contrast to these observations there is evidence that activin B may have specific roles in processes such as embryonic mesoderm induction [Thomsen *et al.*, 1990, *Cell* 63:485-493] and mammary gland development [Robinson *et al.*, 1997, *Development* 124:2701-2708]. Of particular interest is that activin B is presumed to be the activin of relevance in intrapituitary regulation of FSH, as shown by neutralization studies [Corrigan *et al.*, 1991, *Endocrinology* 128:1682-1684]. Additionally, distinct differences in expression patterns of activin A and B are evident during tissue repair [Hübner *et al.*, 1996, *Developmental Biology* 173:490-498] and in association with models of liver fibrosis [De Bleser *et al.*, 1997, *Hepatology,* 26:905-912]. Such evidence suggests that activin A and B play different roles in a range of biological and pathological processes.

Follistatin specifically binds to activin. As a result, circulating follistatin 315 neutralizes activin activity by preventing the interaction of the cytokine with its type II receptors [de Winter *et al.*, *Molecular and Cellular Endocrinology* 1996 116:105–114] and, furthermore, cell surface-bound follistatin 288 facilitates the lysosomal degradation of activin [Hashimoto *et al.*, *Journal of Biological Chemistry* 1997 272:13835–13842]. Both follistatin and activin mRNAs show a broad tissue distribution [Meunier *et al.*, *PNAS* 1988 85:247–251; Michel *et al.*, *Biochemical and Biophysical Research Communications* 1990 173:401–407; Schneider *et al.*, *European Journal of Endocrinology* 2000 142:537–544]. Follistatin and activin are detectable in serum [Demura *et al.*, *Journal of Clinical Endocrinology and Metabolism* 1993 76:1080–1082; Demura *et al.*, *Biochemical and Biophysical Research Communications* 1992 185:1148–1154; Gilfillan *et al.*, *Clinical Endocrinology* 1994 41:453–461; Khoury *et al.*, *Journal of Clinical Endocrinology and Metabolism* 1995 80:1361–1368; Knight *et al.*, *Journal of Endocrinology* 1996 148:267–279; McFarlane *et al.*, *European Journal of Endocrinology* 1996 134:481–489; Sakai *et al.*, *Biochemical and Biophysical Research Communications* 1992 188:921–926; Sakamoto *et al.*, *European Journal of Endocrinology* 1996 135:345–351; Tilbrook *et al.*, *Journal of Endocrinology* 1996 149:55–63; Wakatsuki *et al.*, *Journal of Clinical Endocrinology and Metabolism* 1996 81:630–634], and their concentrations in serum increase with age [Wakatsuki *et al.* 1996, *supra*; Loria *et al.*, *European Journal of Endocrinology* 1998 139:487–492]. At present, however, the precise sources of follistatin and activin in serum are unknown. Current data suggest that tissue-specific balances of follistatin and activin govern the growth and differentiation of responsive cell types in an autocrine/paracrine manner [Michel *et al.*, *Acta Endocrinologica* 1993 129:525–531; Phillips, *Trends in Endocrinology and Metabolism* 2001 12:94–96].

An emerging roll for activin and follistatin in the body's innate immune response has been documented. For instance, activin and follistatin are secreted by various cell types in response to inflammatory compounds *in vitro* [Hübner *et al.*, *Experimental Cell Research* 1996 **228** 106–113; Jones *et al.*, *Endocrinology* 2000 **141** 1905–1908; Keelan *et al.*, *Placenta* 2000 **21** 38–43; Michel *et al.*, *Endocrinology* 1996 **137** 4925–4934; Phillips *et*

al., *Journal of Endocrinology* 1998 **156** 77–82; Yu *et al.*, *Immunology* 1996 **88** 368–374; Erämaa *et al.*, *Journal of Experimental Medicine* 1992 **176** 1449–1452; Shao *et al.*, *Cytokine* 1998 **10** 227–235; Mohan *et al.*, *European Journal of Endocrinology* 2001 **145** 505–511]. Moreover, in some examples of inflammatory processes such as wound healing, inflammatory bowel disease and rheumatoid arthritis, increased activin and/or follistatin expression has been noted [Hübner *et al.*, *Laboratory Investigation* 1997 **77** 311–318; Hübner *et al.*, *Developmental Biology* 1996 **173** 490–498; Yu *et al.*, *Clinical and Experimental Immunology* 1998 **112** 126–132]. However, since these very early and preliminary findings, the role of activin and follistatin in the context of inflammation, *per se*, has not been further elucidated, either in the context of their precise activities or in the context of the scope of the inflammatory conditions in which they function. In light of the extreme diversity in terms of the nature and extent of inflammatory responses which can occur, and the extremely pleiotropic activities of cytokines such as activin, it is not surprising that the preliminary findings of the mid to late 1990's have not progressed to more substantial theories. In particular, activin and follistatin are expressed by a wide variety of cell types and most organs in the body in response to a wide range of stimuli. Accordingly, their usefulness as a marker of inflammation would therefore not be expected.

In sheep models of acute inflammatory challenge, activin and follistatin have been found to be elevated in the blood. However, until now there has been no human data to suggest that activin or follistatin are useful predictors of clinically important inflammatory diseases such as sepsis. While some workers have looked at levels of for instance, follistatin, in inflammatory conditions there has been no recognition until now that examination of those levels can provide useful information of the management of patients with inflammatory conditions. Thus for instance Michel *et al.*, *supra*, although demonstrating that follistatin is elevated in septicemia, did not find a useful correlation with outcome/prognosis. Further, Michel et al, *supra*, demonstrated that follistatin is elevated in meningitis but did not find that this was correlated directly as a clinical indicator.

In work leading up to the present invention it has been surprisingly determined that activin β_B is in fact an accurate, reliable and extremely sensitive diagnostic/prognostic indicator of the onset and severity of an inflammatory response. In fact, it has been surprisingly determined that activin B levels are substantially more highly upregulated following inflammatory stimulation than the corresponding activin A levels. These findings are particularly surprising in light of what has been known to date in relation to the distinct roles of activins A and B. Still further, whereas immunoassays directed to the measurement of activin A have been available for use for some time, analysis of activin B has been inhibited by the absence of a specific immunoassay for this particular activin species. A very limited data set is available which suggests that circulating activin B levels alter during pregnancy or with ovarian function [Petraglia *et al.*, 1993, *Endocrine Journal* 1:323-327; Woodruff *et al.*, 1997, *Journal of Endocrinology* 152:167-174; Vihko *et al.*, 1998, *Human Reproduction* 13:841-846; Vihko *et al.*, 2003, *Acta Obstetricia et Gynecologica Scandinavica,* 80:570-574]. Kobayashi *et al.* (2000) demonstrated that an increase in activin-β_B mRNA is associated with liver regeneration and the development of fibrosis, although the authors do not postulate whether this is linked with changes to levels of activin AB, Activin B or inhibin. A study by Rosendahl *et al.* 2001 examined a mouse model of allergen-induced airway challenge in the lung and focussed on examining associated changes in expression and distribution of TGF-β superfamily and TGF-β/activin receptors. This group reported that induced airway allergens produced only a very modest elevation of activin β_B mRNA expression over control levels. Histological examinations did not provide any information on mature activin dimer protein synthesis or distribution (either activin A or B) nor was there any evidence provided that the modest increase in activin-β_B mRNA levels was not, in fact, linked to changes in inhibin levels. Accordingly, the determination that activin B levels are in fact dramatically increased during inflammation relative to activin A levels is extremely unexpected in light of the very limited information which was available about the functioning of both the activin A and activin B molecules.

These findings have now facilitated the development of diagnostic methodology directed to diagnosing, prognosing and/or monitoring the onset of inflammatory responses or

conditions characterised by an inflammatory response. This now provides means of effectively managing patients exhibiting inflammatory conditions, or predispositions thereto, by providing both a more sensitive tool for screening for and monitoring an inflammatory response and an additional tool for use instead of or together with existing diagnostic methods, such as those based on monitoring activin and/or follistatin levels.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

The subject specification contains nucleotide sequence information prepared using the programme PatentIn Version 3.1, presented herein after the bibliography. Each nucleotide sequence is identified in the sequence listing by the numeric indicator <210> followed by the sequence identifier (eg. <210>1, <210>2, etc). The length, type of sequence (DNA, etc) and source organism for each nucleotide sequence is indicated by information provided in the numeric indicator fields <211>, <212> and <213>, respectively. Nucleotide sequences referred to in the specification are identified by the indicator SEQ ID NO: followed by the sequence identifier (eg. SEQ ID NO:1, SEQ ID NO:2, etc.). The sequence identifier referred to in the specification correlates to the information provided in numeric indicator field <400> in the sequence listing, which is followed by the sequence identifier (eg. <400>1, <400>2, etc). That is SEQ ID NO:1 as detailed in the specification correlates to the sequence indicated as <400>1 in the sequence listing.

One aspect of the present invention is directed to a method for detecting the onset or a predisposition to the onset of an inflammatory response in a mammal, said method comprising screening for the level of activin protein, which activin comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression is indicative of an inflammatory response.

Another aspect of the present invention is directed to a method of detecting the onset or a predisposition to the onset of an inflammatory response in a mammal, said method comprising screening for the level of one or both of activin B protein and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression is indicative of an inflammatory response.

In still another aspect there is provided a method of detecting the onset or a predisposition to the onset of a local inflammatory response in a mammal, said method comprising screening for the level of activin protein, which activin comprises a β_B subunit, and/or gene expression wherein an increase in the level of said protein and/or gene expression is indicative of said local inflammatory response.

In yet another aspect there is provided a method of detecting the onset or a predisposition to the onset of a systemic inflammatory response in a mammal, said method comprising screening for the level of activin protein, which activin comprises a β_B subunit, and/or gene expression wherein an increase in the level of said protein and/or gene expression is indicative of said systemic inflammatory response.

In still yet another aspect there is provided a method of detecting the onset or a predisposition to the onset of an acute systemic inflammatory response in a mammal, said method comprising screening for the level of activin protein, which activin comprises a β_B subunit, and/or gene expression in a mammal wherein an increase in the level of said protein and/or gene expression is indicative of said acute systemic inflammatory response.

In yet still another aspect the present invention relates to a method for monitoring the progression of an inflammatory response in a mammal, said method comprising screening for modulation of the level of activin protein, which activin comprises a β_B subunit, and/or gene expression in said mammal.

A further aspect of the present invention provides a method for monitoring the progression of a localised inflammatory response in a mammal, said method comprising screening for modulation of the level of activin B protein and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression relative to a previously obtained level is indicative of the maintenance or worsening of said response and a decrease in said level is indicative of an improvement in said inflammatory response.

In another further aspect there is provided a method for monitoring the progression of a systemic inflammatory response in a mammal, said method comprising screening for modulation of the level of activin B protein and/or gene expression relative to a previously obtained level wherein an increase in the level of said protein and/or gene expression in said mammal is indicative of the maintenance or worsening of said response and a decrease in said level is indicative of an improvement in said response.

Yet still another further aspect of the present invention is directed to a method for detecting the onset or a predisposition to the onset of a condition characterised by an inflammatory response in a mammal, said method comprising screening for the level of activin protein, which activin comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression is indicative of the onset or predisposition to the onset of said condition.

In still yet another further aspect there is provided a method for detecting the onset or a predisposition to the onset of a condition characterised by an inflammatory response in a mammal, said method comprising screening for the level of activin B protein and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression is indicative of the onset or a predisposition to the onset of said condition.

Yet another aspect is directed to a method for monitoring the progression of a condition characterised by an inflammatory response in a mammal, said method comprising screening for modulation of the level of activin protein, which activin comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression relative to a previously obtained level is indicative of the maintenance or worsening of said condition and a decrease in said level is indicative of an improvement in said condition.

More particularly, there is provided a method for monitoring the progression of a condition characterised by an inflammatory response in a mammal, said method comprising screening for modulation of the level of activin B protein and/or gene expression in said

mammal wherein an increase in the level of said protein and/or gene expression relative to a previously obtained level is indicative of the maintenance or worsening of said condition and a decrease in said level is indicative of an improvement in said condition.

Another aspect of the present invention provides a diagnostic kit for assaying biological samples comprising an agent for detecting the marker proteins or encoding nucleic acid molecules and reagents useful for facilitating the detection by the agent in the first compartment. Further means may also be included, for example, to receive a biological sample. The agent may be any suitable detecting molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Quantitative mRNA levels for activin β_A (upper panel) and βB (lower panel) subunits in livers of mice challenged with a single intraperitoneal injection of LPS. Mice were either treated with LPS alone (no follistatin pretreatment, solid circles) or 1 μg of human recombinant follistatin 288 thirty minutes before LPS (follistatin pretreatment, open circles). Data are represented as mean ± SEM at each timepoint assessed relative to LPS, with expression levels expressed relative to the expression of the housekeeping gene, GADPH. All time 0 data were normalized to a value of 1 and data at subsequent time points were expressed relative to that time point.

Figure 2: Quantitative mRNA levels for activin β_A (upper panel) and βB (lower panel) subunits in livers of mice challenged with a single intraperitoneal injection of CCl_4. Data are represented as mean ± SEM at each timepoint assessed relative to CCl_4, with expression levels expressed relative to the expression of the housekeeping gene, GADPH. All time 0 data were normalized to a value of 1 and data at subsequent time points were expressed relative to that time point.

Figure 3: Immunolocalization of the activin β_A subunit in livers of mice at various timepoints following LPS treatment. The activin β_A subunit was localized to hepatocytes in untreated animals (t=0 hr) but predominantly around the central hepatic veins. Immunolocalization appeared diminished at 5 hours following LPS challenge, but returned to pre-treatment localization patterns by 12 hours (X 50).

Figure 4: Immunolocalization of the activin βB subunit in livers of mice at various timepoints following LPS treatment. The activin βB subunit was localized to hepatocytes in untreated animals (t=0 hr), in areas surrounding portal tracts but not central veins. Immunolocalization appeared diminished at 5 hours following LPS challenge, but returned to pre-treatment localization patterns by 12 hours. Note also the loss of localization in peripheral hepatocytes (asterisks) (X 50).

Figure 5: Immunolocalization of the activin β_A subunit (panels a and b) and activin βB subunit (panels c and d) in livers of mice at 0 or 36 hours following CCl_4 challenge. As for LPS treatment, the activin βB subunit was localized to areas surrounding the portal tract but not central veins whereas the activin β_A subunit predominantly localized to hepatocytes surrounding central veins. Note also the localization of activin β_A subunit at the 36 hour timepoint in areas of hepatocyte apoptosis/necrosis, while localization for the activin β_B subunit is absent from these areas (asterisks) (X50).

Figure 6: Western blot analysis of activin βB subunit in mouse serum samples, with molecular weights of protein standards indicated on the left side. Lane 1, conditioned medium from 293 cells overexpressing the activin βB subunit (positive control); Lane 2, mouse serum at t=0 hours (untreated); Lane 3, mouse serum at t=0 hours (untreated), Lane 4, mouse serum at t=5 hours following LPS injection; Lane 5, mouse serum at t=5 hours following LPS injection; Lane 6, mouse serum at t=36 hours following CCl_4 injection.

Figure 7: Localisation of the activin β_B subunit in tissue sections from burns injury patients, with brown colouring (diaminobenzidine) present in immunopositive structures. The counterstain (blue colouring) is haematoxylin. **(A)** and **(B)** Localisation was predominantly in the stratum granulosum (SGr) and the stratum spinosum (SS) layers but not in the stratum lucidum (SL) or the stratum germinativum (SGe). The inset in panel (A) is a negative control section with no activin β_B antibody applied ; **(C)** Localization in the basal layers of hair follicles (HF); **(D)** Localisation in the basal layers of sebaceous glands (SG); **(E)** Mild staining for activin β_B in the secretory elements of sweat glands (SwtGld); **(F)** Strong localization in infiltrating immune cells indicated by immunopositive rounded cells; **(G)** Localisation was also present in the endothelium of blood vessels (BV). Magnifications: Panel A (including inset) and G, 4x, Panels B-F, 20x.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated, in part, on the determination that activin molecules comprising a β_B subunit, in particular activin B, are accurate and highly sensitive indicators of both the onset or predisposition to the onset of an inflammatory response and the likely severity of such a response. Specifically, activin molecules comprising the β_B subunit have been found to regulate the very early stages of the inflammatory response, despite otherwise exhibiting significant functional distinctiveness relative to activin A. However most surprisingly, they have been found to exhibit significantly higher levels of expression than activin A. Accordingly, the present invention provides a means of assessing an inflammatory response, in particular a systemic inflammatory response, based on relative levels of activin molecules comprising a β_B subunit, in particular activin B. These findings have therefore facilitated the development of a highly sensitive and informative assay directed to diagnosing the onset or predisposition to the onset of an inflammatory response or a condition characterised by an inflammatory response.

Accordingly, one aspect of the present invention is directed to a method for detecting the onset or a predisposition to the onset of an inflammatory response in a mammal, said method comprising screening for the level of an activin protein, which protein comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression is indicative of an inflammatory response.

Reference to "an activin protein, which activin protein comprises a β_B subunit" should be understood as a reference to a monomeric or multimeric molecule, preferably a dimer, which comprises at least one activin β_B subunit. Reference to "activin β_B" should be understood as a reference to all forms of activin β_B and to fragments, derivatives, mutants or variants thereof. "Activin β_B subunit" is also interchangeably referred to as "activin β_B". It should be understood to include reference to any isoforms which may arise from alternative splicing of activin β_B mRNA or mutant or polymorphic forms of activin β_B. Reference to "activin β_B" is not intended to be limiting and should be read as including reference to all forms of activin β_B including any protein encoded by the activin β_B subunit

gene, any subunit polypeptide such as precursor forms which may be generated, and any activin β_B protein, whether existing as a monomer, multimer or fusion protein. Multimeric protein forms of activin β_B include for example the homodimeric activin B (β_B-β_B) or the heterodimeric activin AB (β_A-β_B), activin BC (β_B-β_C), activin BD (β_B-β_D) or activin BE (β_B-β_E) proteins. Preferably, said activin molecule is activin B.

More particularly, the present invention is directed to a method of detecting the onset or a predisposition to the onset of an inflammatory response in a mammal, said method comprising screening for the level of activin B protein and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression is indicative of an inflammatory response.

Without limiting the present invention to any one theory or mode of action, the inflammatory response is a complex response characterised by a series of physiological and/or immunological events which are induced to occur by the release of a cytokine cascade in response to any one of a variety of stimuli including, but not limited to, tissue injury, infection, an immune response (such as to a pathogen or an innocuous agent – as occurs with allergies), or disease (such as tumour formation or an autoimmune response).

The physiological events which characterise inflammation include:

(i) vasodilation
(ii) increased vascular permeability
(iii) cellular infiltration
(iv) changes to the biosynthetic, metabolic and catabolic profiles of affected organs
(v) activation of the cells of the immune system.

It should be understood that reference to an "inflammatory response" is a reference to any one or more of the physiological and/or immunological events or phases that are induced to occur in the context of inflammation and, in general, in response to the signals generated by the cytokine cascade which largely directs an inflammatory response. For example IL-

1, TNFα and IL-6 are well known for their functions as pro-inflammatory mediators. It should also be understood that an inflammatory response within the context of the present invention essentially includes a reference to a partial response, such as a response which has only just commenced, or to any specific phase or event of a response (such as the phases and events detailed in points (i)-(v), above, or any other effect related to inflammation including, but not limited to, the production of acute phase proteins – including complement components, fever and a systemic immune response). Further, it should also be understood that depending on any given set of specific circumstances, the end point of an inflammatory response may vary. For example, in some situations there may only occur an acute vascular response. To the extent that "acute" inflammation occurs, this is generally understood to include the events of both an acute vascular response and an acute cellular response. Some inflammatory responses will resolve at the acute stage while others may progress to become chronic cellular responses.

Without limiting the present invention to any one theory or mode of action, in certain circumstances the acute process, characterized by neutrophil infiltration and oedema, gives way to a predominance of mononuclear phagocytes and lymphocytes. This is thought to occur to some degree with the normal healing process but becomes exaggerated and chronic when there is ineffective elimination of foreign materials as in certain infections (e.g. tuberculosis) or following introduction of foreign bodies (e.g. asbestos) or deposition of crystals (e.g. urate crystals). Chronic inflammation is often associated with fusion of mononuclear cells to form multinucleated gigant cells, which eventually become a granuloma. Chronic inflammation is also seen under conditions of delayed hypersensitivity. The subject inflammatory response may be systemic or localised. Examples of systemic inflammatory responses include those which fall within the scope of systemic inflammatory response syndrome such as septic shock, toxic shock or septicaemia.

Examples of localised inflammatory responses include those which occur in the context of rheumatoid arthritis, inflammatory bowel disease, pancreatitis, atherosclerosis, meningitis, appendicitis, angiogenesis, psoriasis, neural protection, renal tubular necrosis, myocardial

infarction, allergic responses and wound healing (for example, pursuant to surgery, burns or other tissue injury). It should be understood, however, that some localised inflammatory responses can become systemic, for example as can occur when the onset of septic shock occurs as a complication of severe burns or abdominal wounds. In another example, septicaemia can result from the transition of a more localised bacterial infection to a circulatory infection.

Accordingly, in one preferred embodiment there is provided a method of detecting the onset or a predisposition to the onset of a local inflammatory response in a mammal, said method comprising screening for the level of an activin protein, which protein comprises a β_B subunit, and/or gene expression wherein an increase in the level of said protein and/or gene expression is indicative of said local inflammatory response.

Preferably, said activin is activin B.

More preferably, said local inflammatory response is acute.

In another preferred embodiment there is provided a method of detecting the onset or a predisposition to the onset of a systemic inflammatory response in a mammal, said method comprising screening for the level of an activin protein, which protein comprises a β_B subunit, and/or gene expression wherein an increase in the level of said protein and/or gene expression is indicative of said systemic inflammatory response.

Preferably, said activin is activin B.

More preferably, said systemic inflammatory response is acute.

According to this most preferred embodiment there is provided a method of detecting the onset or a predisposition to the onset of an acute systemic inflammatory response in a mammal, said method comprising screening for the level of activin B protein and/or gene

expression in a mammal wherein an increase in the level of said protein and/or gene expression is indicative of said acute systemic inflammatory response.

In accordance with these preferred aspects of the present invention, said acute inflammatory response occurs in the context of, or is otherwise associated with, septic shock, septicaemia, appendicitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, or wound healing or traumatic injury such as occurs with surgery and burns.

Preferably, said acute systemic inflammatory response occurs in the context of systemic inflammatory response syndrome and even more particularly sepsis, septicaemia, toxic shock, septic shock, tissue trauma, meningitis or appendicitis.

The term "mammal" as used herein includes humans, primates, livestock animals (eg. horses, cattle, sheep, pigs, donkeys), laboratory test animals (eg. mice, rats, guinea pigs), companion animals (eg. dogs, cats) and captive wild animal (eg. kangaroos, deer, foxes). Preferably, the mammal is a human or a laboratory test animal. Even more preferably, the mammal is a human.

The present invention is predicated on the determination that activin B levels become increased in the context of an inflammatory response. Without limiting the present invention to any one theory or mode of action, in one example of a model of acute systemic inflammation, activin βA subunit mRNA showed a minor increase (<2-fold control levels) in expression level 1 hour after stimulation of an inflammatory response via LPS. In contrast, liver βB subunit mRNA levels displayed a completely different profile to the activin βA subunit, rising immediately after stimulation of the inflammatory response to reach a maximal expression level at 5 hours, at which time expression averaged over 35-fold control levels. Between 5 and 12 hours this expression fell progressively but at 12 hours, expression was still elevated (on average, 7-fold control levels). At 24 hours activin βB mRNA levels were still ~5- fold above control levels.

Still without limiting the present invention to any one theory or mode of action, it has been determined that early within the onset of an inflammatory response, activin βB levels are increased. This is accompanied by the release of a cascade of cytokines including TNFα, IL-6 and follistatin. Accordingly, activin βB is one of the earlier cytokines released subsequently to an inflammatory response stimulus. The method of the present invention can therefore detect both the onset of an inflammatory response and, to the extent that inflammation-related symptoms are not yet evident, a predisposition to the development of an inflammatory response since the upregulation of activin βB is indicative of the forthcoming development of one or more phases or events of an inflammatory response. Reference to "detecting" an inflammatory response should therefore be understood in its broadest context and includes, *inter alia*, diagnosing, screening, confirming or otherwise assessing an inflammatory response or a condition characterised by the onset of an inflammatory response.

The method of the present invention is predicated on the correlation of activin B in individuals with normal levels of these molecules. The "normal level" is the level of activin comprising a β_B subunit in a corresponding biological sample of a subject who has not developed an inflammatory response nor is predisposed to the development of an inflammatory response in the context described above. Without limiting the present invention in any way, it is generally believed that the systemic level of activin B, to the extent that one is screening at the systemic level, in a normal individual will be negligible. However, activin B levels have been found to increase somewhat with gestational age.

Accordingly, the term "modulation" refers to increases and decreases in activin B levels relative either to a normal reference level (or normal reference level range) or to an earlier activin B level result determined from the subject. A normal reference level is the activin B level from a relevant biological sample of a subject or group of subjects which are not experiencing an inflammatory response. In a preferred embodiment, said normal reference level is the level determined from one or more subjects of a relevant cohort to that of the subject being screened by the method of the invention. By "relevant cohort" is meant a cohort characterised by one or more features which are also characteristic of the subject

who is the subject of screening. These features include, but are not limited to, age, gender, ethnicity or health status, for example.

This reference level may be a discrete figure or may be a range of figures. The reference level may vary between individual classes of molecules which comprise an activin β_B subunit. For example, the normal level of activin β_B homodimer may differ to the normal level of activin β_B heterodimers.

Preferably, said inflammatory response is an acute response and said increase in activin B levels is at least 5 fold over levels in the normal range, preferably 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 or more fold over levels in the normal range. Most preferably, said activin B level increase is at least 7 fold over levels in the normal range and still more preferably at least 35 fold over levels in the normal range.

Although the preferred method is to detect an increase in activin B levels in order to diagnose the onset or a predisposition to the onset of an inflammatory response, the detection of a decrease in the levels of these molecules may be desired under certain circumstances. For example, to monitor improvement in the status of an inflammatory response during the course of prophylactic or therapeutic treatment of patients presenting with an acute or chronic inflammatory response or a condition associated with such a response, such as sepsis, septicaemia, meningitis, rheumatoid arthritis, or a tissue trauma. Further, although upregulation in the levels of these molecules will generally be regarded as adverse, since it is likely to be indicative of an unwanted inflammatory response, in some situations one may be screening for the induction of a *desired* inflammatory response such as where an inflammatory response is designed to provide adjuvant-like activity. This may be particularly useful in the context of anti-tumour therapy. In still another example, the upregulation of host defence mechanisms may be desired.

This aspect of the present invention also enables one to monitor the progression of an inflammatory response or a condition characterised by an inflammatory response. By

"progression" is meant the ongoing nature of an inflammatory response, such as its improvement, maintenance, worsening or a change in the level of its severity.

Accordingly, another aspect of the present invention relates to a method for monitoring the progression of an inflammatory response in a mammal, said method comprising screening for modulation of the level of activin protein, which protein comprises a β_B subunit, and/or gene expression in said mammal.

Preferably, said activin is activin B.

In one particularly preferred embodiment, said inflammatory response is an acute localised inflammatory response or an acute systemic inflammatory response.

In accordance with these preferred aspects of the present invention, said acute inflammatory responses occurs in the context of, or is otherwise associated with, septic shock, septicaemia, appendicitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, myocardial infarction or wound healing or traumatic injury such as occurs with surgery and burns.

Most preferably, said acute systemic inflammatory response occurs in the context of systemic inflammatory response syndrome and even more particularly sepsis, toxic shock, septic shock, septicaemia, tissue trauma, meningitis or appendicitis.

It should be understood that in accordance with this aspect of the present invention, activin B levels will likely be assessed relative to one or more previously obtained levels from the patient in issue.

One particularly preferred embodiment of the present invention therefore provides a method for monitoring the progression of a localised inflammatory response in a mammal, said method comprising screening for modulation of the level of activin protein, which

protein comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression relative to a previously obtained level is indicative of the maintenance or worsening of said response and a decrease in said level is indicative of an improvement in said inflammatory response.

Preferably, said activin is activin B.

Preferably, said inflammatory response is an acute response.

In another particularly preferred embodiment there is provided a method for monitoring the progression of a systemic inflammatory response in a mammal, said method comprising screening for modulation of the level of activin protein, which protein comprises a β_B subunit, and/or gene expression relative to a previously obtained level wherein an increase in the level of said protein and/or gene expression in said mammal is indicative of the maintenance or worsening of said response and a decrease in said level is indicative of an improvement in said response.

Preferably, said activin is activin B.

Preferably, said inflammatory response is a systemic response.

The method of the present invention has widespread application including, but not limited to the diagnostic/prognostic analysis of an inflammatory response or inflammatory response symptoms or aspects of any condition characterised by the presence of an inflammatory response such as septic shock, septicaemia, appendicitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, myocardial infarction or wound healing or traumatic injury such as occurs with surgery and burns.

Accordingly, another aspect of the present invention is directed to a method for detecting the onset or a predisposition to the onset of a condition characterised by an inflammatory

response in a mammal, said method comprising screening for the level of activin protein, which protein comprises a β_B subunit, and/or gene expression in said mammal where an increase in the level of said protein and/or gene expression is indicative of the onset or predisposition to the onset of said condition.

More particularly, there is provided a method for detecting the onset or a predisposition to the onset of a condition characterised by an inflammatory response in a mammal, said method comprising screening for the level of activin protein, which protein comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression is indicative of the onset or a predisposition to the onset of said condition.

Yet another aspect is directed to a method for monitoring the progression of a condition characterised by an inflammatory response in a mammal, said method comprising screening for modulation of the level of activin protein, which protein comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression relative to a previously obtained level is indicative of the maintenance or worsening of said condition and a decrease in said level is indicative of an improvement in said condition.

More particularly, there is provided a method for monitoring the progression of a condition characterised by an inflammatory response in a mammal, said method comprising screening for modulation of the level of activin protein, which protein comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression relative to a previously obtained level is indicative of the maintenance or worsening of said condition and a decrease in said level is indicative of an improvement in said condition.

In yet another aspect there is provided a method for assessing the severity of a condition characterised by an inflammatory response in a mammal, said method comprising quantitatively screening for the level of activin protein, which protein comprises a β_B

subunit, and/or gene expression in said mammal wherein the degree of increase in the level of said protein and/or gene expression is indicative of the severity of said condition.

Preferably, said activin is activin B.

In accordance with these aspects of the present invention, said inflammatory response is preferably an acute localised inflammatory response or an acute localised response.

In accordance with these preferred aspects of the present invention, said acute inflammatory responses occur in the context of, or is otherwise associated with, septic shock, toxic shock, sepsis, septicaemia, appendicitis, pancreatitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, myocardial infarction or wound healing or traumatic injury such as occurs with surgery and burns.

Most preferably, said acute systemic inflammatory response occurs in the context of systemic inflammatory response syndrome and even more particularly sepsis, septic shock, toxic shock, septicaemia, tissue trauma, meningitis or appendicitis.

It should be understood that although the present invention is directed to screening for levels of activin B, this screening test need not necessarily constitute the only test which is performed in the context of a given patient. For example, one may seek to also pursue additional tests such as analysis of activin A and/or follistatin levels. In this way, a profile may be generated in relation to a particular patient, thereby providing very detailed diagnostic and/or prognostic information. Still further, the generation of such profiles provides a highly sensitive framework within which to monitor a patient.

It should also be understood that the screening methodology herein defined may be performed either quantitatively or qualitatively. Although it is likely that quantitative analyses will be preferred since they provide information in relation to both the existence, or not, of an inflammatory condition in addition to identifying its severity, the method of

the present invention does facilitate qualitative analyses. In particular, since activin B is usually not found in the blood in appreciable amounts, to the extent that systemic analysis is being performed a test directed to assessing the presence or not of activin will provide useful information. It will also provide scope for establishing extremely simple and inexpensive screening procedures.

Methods of screening for levels of activin can be achieved by any suitable method which would be well known to persons of skill in the art. In this regard, it should be understood that reference to screening for the level of protein and/or gene expression "in a mammal" is intended as a reference to the use of any suitable technique which will provide information in relation to the level of expression of activin in the relevant tissue of the mammal. These screening techniques include both *in vivo* screening techniques, as hereinafter described, as well as the *in vitro* techniques which are applied to a biological sample extracted from said mammal. Such *in vitro* techniques are likely to be preferred due to their significantly more simplistic and routine nature.

Since the present invention is predicated on screening for changes in the level of, preferably, activin B, such changes can in fact be screened for at the protein level or at the nucleic acid level, such as by screening for increases in the level of activin B mRNA transcripts. The person of skill in the art will determine the most appropriate means of analysis in any given situation. However it is generally preferred that screening be performed in the context of protein molecules due to the relative simplicity of the techniques which are likely to be utilised. Nevertheless in certain situations, and in the context of particular biological samples, it may be desirable or otherwise useful to directly analyse gene transcription.

Still further, to the extent that one is analysing a biological sample harvested from a patient, it is within the skill of the person in the art to determine the most appropriate sample for analysis. For example, blood components are likely to provide a most convenient means for analysing systemic levels of activin protein levels in the context of systemic inflammatory responses. However, to the extent that one is assessing potential

localised inflammatory responses, other types of biological samples may be more suitable. For example, early stage rheumatoid arthritis may be initially assessed by determining the presence or level of inflammatory response by analysing levels of activin B in the synovial fluid of an affected joint. Similarly, suspected meningitis may be assessed in terms of the degree of inflammatory response by analysing the spinal fluid which is generally automatically harvested from a patient for the purpose of a range of routine analytical tests which are performed. In other situations, it may be more appropriate to analyse biopsy specimens.

Reference to a "biological sample" should therefore be understood as a reference to any sample of biological material derived from an individual such as, but not limited to, mucus, stool, urine, blood, serum, central nervous system fluid (eg. spinal fluid), cell extract, biopsy specimens (eg. heart tissue) and fluid which has been introduced into the body of an individual and subsequently removed such as, for example, the saline solution extracted from the lung following lung lavage or the solution retrieved from an enema wash. The biological sample which is tested according to the method of the present invention may be tested directly or may require some form of treatment prior to testing. For example, a biopsy sample may require homogenisation or sectioning prior to testing.

In a preferred embodiment, the subject inflammatory response which is under investigation is a systemic inflammatory response and the biological sample which is subjected to analysis is a blood sample, or a component of a blood sample. Most preferably, the protein form of activin β_B is screened for.

As described above, means of screening for changes in levels of activin molecules which comprise the activin β_B subunit (herein referred to as "the markers") in an individual, or biological sample derived therefrom, can be achieved by any suitable method, which would be well known to the person of skill in the art, such as but not limited to:

(i) *In vivo* detection of the markers. Molecular Imaging may be used following administration of imaging probes or reagents capable of disclosing altered

expression levels of the markers mRNA or protein expression product in the prostate tissues.

Molecular imaging (Moore, A., Basilion, J., Chiocca, E., and Weissleder, R., *BBA, 1402:*239-249, 1988; Weissleder, R., Moore, A., Ph.D., Mahmood-Bhorade, U., Benveniste, H., Chiocca, E.A., Basilion, J.P. *Nature Medicine, 6:*351-355, 2000) is the *in vivo* imaging of molecular expression that correlates with the macro-features currently visualized using "classical" diagnostic imaging techniques such as X-Ray, computed tomography (CT), MRI, Positron Emission Tomography (PET) or endoscopy. Historically, detection of malignant tumor cells in a background of normal or hyperplastic benign tissue is often based on differences in physical properties between tissues, which are frequently minimal, resulting in low contrast resolution. Application of expression profiling will define the differences in "molecular properties" between cancer and normal tissues that arise as a result of malignant transformation.

(ii) Detection of up-regulation of mRNA expression in the cells by Fluorescent *In Situ* Hybridization (FISH), or in extracts from the cells by technologies such as Quantitative Reverse Transcriptase Polymerase Chain Reaction (QRTPCR) or Flow cytometric qualification of competitive RT-PCR products (Wedemeyer, N., Potter, T., Wetzlich, S. and Gohde, W. *Clinical Chemistry 48:*9 1398-1405, 2002) or array technologies.

For example, a labelled polynucleotide encoding the markers may be utilized as a probe in a Northern blot of an RNA extract obtained from the prostate. Preferably, a nucleic acid extract from the animal is utilized in concert with oligonucleotide primers corresponding to sense and antisense sequences of a polynucleotide encoding the markers, or flanking sequences thereof, in a nucleic acid amplification reaction such as RT PCR, real time PCR or SAGE. A variety of automated solid-phase detection techniques are also appropriate. For example, a very large scale immobilized primer arrays (VLSIPS™) are used for the detection of nucleic acids

as, for example, described by Fodor *et al.*, 1991 and Kazal *et al.*, 1996. The above genetic techniques are well known to persons skilled in the art.

For example, to detect the markers encoding RNA transcripts, RNA is isolated from a cellular sample suspected of containing the markers RNA, e.g. total RNA isolated from human prostate cancer tissue. RNA can be isolated by methods known in the art, e.g. using TRIZOL™ reagent (GIBCO-BRL/Life Technologies, Gaithersburg, Md.). Oligo-dT, or random-sequence oligonucleotides, as well as sequence-specific oligonucleotides can be employed as a primer in a reverse transcriptase reaction to prepare first-strand cDNAs from the isolated RNA. Resultant first-strand cDNAs are then amplified with sequence-specific oligonucleotides in PCR reactions to yield an amplified product.

"Polymerase chain reaction" or "PCR" refers to a procedure or technique in which amounts of a preselected fragment of nucleic acid, RNA and/or DNA, are amplified as described in U.S. Patent No. 4,683,195. Generally, sequence information from the ends of the region of interest or beyond is employed to design oligonucleotide primers. These primers will be identical or similar in sequence to opposite strands of the template to be amplified. PCR can be used to amplify specific RNA sequences and cDNA transcribed from total cellular RNA. See generally Mullis *et al.*, 1987; Erlich, 1989. Thus, amplification of specific nucleic acid sequences by PCR relies upon oligonucleotides or "primers" having conserved nucleotide sequences wherein the conserved sequences are deduced from alignments of related gene or protein sequences, e.g. a sequence comparison of mammalian the markers genes. For example, one primer is prepared which is predicted to anneal to the antisense strand and another primer prepared which is predicted to anneal to the sense strand of a cDNA molecule which encodes the markers.

To detect the amplified product, the reaction mixture is typically subjected to agarose gel electrophoresis or other convenient separation technique and the relative presence of the markers specific amplified DNA detected. For example, the

markers amplified DNA may be detected using Southern hybridization with a specific oligonucleotide probe or comparing is electrophoretic mobility with DNA standards of known molecular weight. Isolation, purification and characterization of the amplified the markers DNA may be accomplished by excising or eluting the fragment from the gel (for example, see references Lawn *et al.*, 1981; Goeddel *et al.*, 1980), cloning the amplified product into a cloning site of a suitable vector, such as the pCRII vector (Invitrogen), sequencing the cloned insert and comparing the DNA sequence to the known sequence of the markers. The relative amounts of the markers mRNA and cDNA can then be determined.

(iii) Measurement of altered the markers protein levels in cell extracts or blood or other suitable biological sample, either qualitatively or quantitatively, for example by immunoassay, utilising immunointeractive molecules such as monoclonal antibodies.

In one example, one may seek to detect the markers -immunointeractive molecule complex formation. For example, an antibody according to the invention, having a reporter molecule associated therewith, may be utilized in immunoassays. Such immunoassays include but are not limited to radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs) and immunochromatographic techniques (ICTs), Western blotting which are well known to those of skill in the art. For example, reference may be made to "Current Protocols in Immunology", 1994 which discloses a variety of immunoassays which may be used in accordance with the present invention. Immunoassays may include competitive assays. It will be understood that the present invention encompasses qualitative and quantitative immunoassays.

Suitable immunoassay techniques are described, for example, in U.S. Patent Nos. 4,016,043, 4,424,279 and 4,018,653. These include both single-site and two-site assays of the non-competitive types, as well as the traditional competitive binding assays. These assays also include direct binding of a labelled antigen-binding

molecule to a target antigen. The antigen in this case is the markers or a fragment thereof.

Two-site assays are particularly favoured for use in the present invention. A number of variations of these assays exist, all of which are intended to be encompassed by the present invention. Briefly, in a typical forward assay, an unlabelled antigen-binding molecule such as an unlabelled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen complex, another antigen-binding molecule, suitably a second antibody specific to the antigen, labelled with a reporter molecule capable of producing a detectable signal is then added and incubated, allowing time sufficient for the formation of another complex of antibody-antigen-labelled antibody. Any unreacted material is washed away and the presence of the antigen is determined by observation of a signal produced by the reporter molecule. The results may be either qualitative, by simple observation of the visible signal, or may be quantitated by comparing with a control sample containing known amounts of antigen. Variations on the forward assay include a simultaneous assay, in which both sample and labelled antibody are added simultaneously to the bound antibody. These techniques are well known to those skilled in the art, including minor variations as will be readily apparent.

In the typical forward assay, a first antibody having specificity for the antigen or antigenic parts thereof is either covalently or passively bound to a solid surface. The solid surface is typically glass or a polymer, the most commonly used polymers being cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs of microplates, or any other surface suitable for conducting an immunoassay. The binding processes are well known in the art and generally consist of cross-linking covalently binding or physically adsorbing, the polymer-antibody complex is washed in preparation for the test sample. An aliquot of the sample to be tested is

then added to the solid phase complex and incubated for a period of time sufficient and under suitable conditions to allow binding of any antigen present to the antibody. Following the incubation period, the antigen-antibody complex is washed and dried and incubated with a second antibody specific for a portion of the antigen. The second antibody has generally a reporter molecule associated therewith that is used to indicate the binding of the second antibody to the antigen. The amount of labelled antibody that binds, as determined by the associated reporter molecule, is proportional to the amount of antigen bound to the immobilized first antibody.

An alternative method involves immobilizing the antigen in the biological sample and then exposing the immobilized antigen to specific antibody that may or may not be labelled with a reporter molecule. Depending on the amount of target and the strength of the reporter molecule signal, a bound antigen may be detectable by direct labelling with the antibody. Alternatively, a second labelled antibody, specific to the first antibody is exposed to the target-first antibody complex to form a target-first antibody-second antibody tertiary complex. The complex is detected by the signal emitted by the reporter molecule.

From the foregoing, it will be appreciated that the reporter molecule associated with the antigen-binding molecule may include the following:-

(a) direct attachment of the reporter molecule to the antibody;

(b) indirect attachment of the reporter molecule to the antibody; i.e., attachment of the reporter molecule to another assay reagent which subsequently binds to the antibody; and

(c) attachment to a subsequent reaction product of the antibody.

The reporter molecule may be selected from a group including a chromogen, a catalyst, an enzyme, a fluorochrome, a chemiluminescent molecule, a paramagnetic ion, a lanthanide ion such as Europium (Eu^{34}), a radioisotope including other nuclear tags and a direct visual label.

In the case of a direct visual label, use may be made of a colloidal metallic or non-metallic particle, a dye particle, an enzyme or a substrate, an organic polymer, a latex particle, a liposome, or other vesicle containing a signal producing substance and the like.

A large number of enzymes suitable for use as reporter molecules is disclosed in U.S. Patent Nos. U.S. 4,366,241, U.S. 4,843,000, and U.S. 4,849,338. Suitable enzymes useful in the present invention include alkaline phosphatase, horseradish peroxidase, luciferase, β-galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzymes may be used alone or in combination with a second enzyme that is in solution.

Suitable fluorochromes include, but are not limited to, fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), R-Phycoerythrin (RPE), and Texas Red. Other exemplary fluorochromes include those discussed by Dower *et al.*, International Publication No. WO 93/06121. Reference also may be made to the fluorochromes described in U.S. Patent Nos. 5,573,909 (Singer *et al*), 5,326,692 (Brinkley *et al*). Alternatively, reference may be made to the fluorochromes described in U.S. Patent Nos. 5,227,487, 5,274,113, 5,405,975, 5,433,896, 5,442,045, 5,451,663, 5,453,517, 5,459,276, 5,516,864, 5,648,270 and 5,723,218.

In the case of an enzyme immunoassay, an enzyme is conjugated to the second antibody, generally by means of glutaraldehyde or periodate. As will be readily recognised, however, a wide variety of different conjugation techniques exist which are readily available to the skilled artisan. The substrates to be used with the specific enzymes are generally chosen for the production of, upon hydrolysis by the

corresponding enzyme, a detectable colour change. Examples of suitable enzymes include those described *supra*. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. In all cases, the enzyme-labelled antibody is added to the first antibody-antigen complex, allowed to bind, and then the excess reagent washed away. A solution containing the appropriate substrate is then added to the complex of antibody-antigen-antibody. The substrate will react with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an indication of the amount of antigen which was present in the sample.

Alternately, fluorescent compounds, such as fluorescein, rhodamine and the lanthanide, europium (EU), may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labelled antibody adsorbs the light energy, inducing a state to excitability in the molecule, followed by emission of the light at a characteristic colour visually detectable with a light microscope. The fluorescent-labelled antibody is allowed to bind to the first antibody-antigen complex. After washing off the unbound reagent, the remaining tertiary complex is then exposed to light of an appropriate wavelength. The fluorescence observed indicates the presence of the antigen of interest. Immunofluorometric assays (IFMA) are well established in the art and are particularly useful for the present method. However, other reporter molecules, such as radioisotope, chemiluminescent or bioluminescent molecules may also be employed.

(iv) The use of aptamers in screening for nucleic acid molecules or expression products

(v) Determining altered protein expression based on any suitable functional test, enzymatic test or immunological test in addition to those detailed in point (iii) - above.

As detailed above, any suitable technique may be utilised to detect the markers or their encoding nucleic acid molecule. The nature of the technique which is selected for use will largely determine the type of biological sample which is required for analysis. Such determinations are well within the scope of the person of skill in the art. Typical samples which one may seek to analyse are biopsy samples of the prostate or blood samples.

Another aspect of the present invention provides a diagnostic kit for assaying biological samples comprising an agent for detecting the marker proteins or encoding nucleic acid molecules and reagents useful for facilitating the detection by the agent in the first compartment. Further means may also be included, for example, to receive a biological sample. The agent may be any suitable detecting molecule.

The present invention is further described by reference to the following non-limiting examples.

EXAMPLE 1

PROFOUND CHANGES IN ACTIVIN β_B DURING LOCALISED AND SYSTEMIC INFLAMMATION

Materials and Methods

Experimental design

For the systemic LPS model, male C57/BL mice were injected intraperitoneally (ip) with 100 μg phenol-purified LPS (Sigma: *E.Coli* (0127:B8). Control mice, injected with PBS, were sacrificed at time 0 and remaining animals (6/time point) at 0.5, 1, 3, 5, 8, 12 and 24 hours and following LPS injection. In an independent experiment, the effects of activins were neutralized by the pretreatment of mice with the activin binding protein, follistatin, which is able to bind and ablate the effects of activin forms [Nakamura *et al.*, 1990, *Science* 247:836-838]. In this experiment, mice were pretreated ip with humar recombinant follistatin 288 (1 μg) 30 minutes prior to an injection of LPS. Mice were sacrificed 30 minutes after the follistatin injection (time 0) and at the same times, relative to LPS, as indicated above. At the time of sacrifice, tissues to be examined for expression levels were placed in ice cold Trizol (Invitrogen Life Technologies) and stored at –80 C for later RNA extraction. Tissues were also placed in formalin prior to transfer to 70% ethanol for later fixation and immunohistochemical studies.

For the acute hepatic inflammation model, male C57/BL6 mice were injected ip with 750 μl/kg BW CCl_4 (Sigma). Control mice, injected with PBS, were sacrificed at time 0 and remaining animals were sacrificed at 1, 2, 4,8, 12, 24, 36, 48 and 72 hours following CCl_4 injection. Tissues were collected as described earlier for RNA extraction and immunohistochemical studies.

RNA extractions were performed on 3-5 tissue samples from each time point described above. RNA was extracted using Trizol according to the manufacturer's recommendations. For each sample, approximately 10 μg of RNA was treated with DNAse I (Ambion Inc.) in

accordance with the manufacturer's protocol. RNA concentrations for each sample were determined and 1 μg was reverse transcribed to give cDNA using Superscript III reverse transcriptase kit (Invitrogen Life technologies) and using the protocol supplied by the manufacturer. Real time analysis for expression levels were made for the following genes: GAPDH, activin β_A subunit, and activin β_B subunit. Inhibin α-subunit mRNA expression was also examined using standard thermocycler methods but expression levels were consistently too low to permit quantitative analysis (data not shown).

The specific primers utilized for the real-time quantification of the genes were (5'- to 3'):

GAPDH	F tactggcatcttcaccacca (Product 394 bp)	(SEQ ID NO:1)
activin β_A	Fggctaacagaaccaggacca (Product 325bp)	(SEQ ID NO:2)
activin β_B	F gacacgcatagccagactca (Product 399bp)	(SEQ ID NO:3)
inhibin α-subunit	F cttatgtattccggccatcc (Product 326bp)	(SEQ ID NO:4)
GAPDH	R gtgagcttcccattcagctc (Product 394 bp)	(SEQ ID NO:5)
activin β_A	R cttcttcccatctccatcca (Product 325bp)	(SEQ ID NO:6)
activin β_B	R acttgccctctccaagaaca (Product 399bp)	(SEQ ID NO:7)
inhibin α-subunit	R cctagtgtgggctaccagga (Product 326bp)	(SEQ ID NO:8)

The primers were designed specifically for use with the Roche light cycler real-time PCR system. PCR products were isolated and sequenced and BLAST analysis used to confirm they represented the desired gene products. Real time analyses were conducted using Roche SYBR green mastermix (Light cycler Fast start DNA Master SYBR green , Roche Diagnostics GmbH) with conditions optimised for maximal sensitivity. Annealing temperatures for all primers were 60 C. Standards and QCs used throughout the analyses were prepared from pooled cDNA derived from experimental samples in which expression levels of the genes of interest were high. Serial dilutions of the standard cDNA was to cover a 300-fold expression range. Experimental cDNA samples were diluted into the standard curve range and all cDNA was aliquoted and stored at –20. Each sample was analysed for all three gene products of interest, at least twice in independent analytical runs. Between assay QC reproducibility for all gene products gave CVs of < 22%.

Immunohistochemistry

Paraffin sections were dewaxed and antigens retrieved by immersing slides in 0.01M citrate buffer, pH 6.0, heating in a microwave (high for 2.5 minutes or 5 minutes for βA or βB respectively, then low for 5 minutes for both), cooling at 4 C for ~20 minutes, and washing in water for 5 minutes. Endogenous peroxidase was blocked in 3% H_2O_2 for 10 minutes, and slides blocked for 1 hour (10% normal rabbit serum + CAS block, Zymed Laboratories Inc., CA, # 00-8120) for activin βA or 20% normal goat serum/0.1% Tween 20 in Tris-buffered saline (TBS) for 1 hour for activin βB. The blocking solution was tapped off and the sections were incubated with antibodies specific for the activin βA-subunit (E4, 10 μg/ml in 1% bovine serum albumin (BSA)/TBS, Oxford Brookes University) or activin βB subunit (2 μg/ml diluted in blocking solution, Jones *et al.* 2000) overnight at 4 C. After washing, the activin βA slides were incubated in rabbit anti-mouse IgG_{2b}-HRP (Zymed, # 61-0320) diluted 1:500 for 2 hours and washed twice in Tris-buffered NaCl (TBS) 0.05% Tween-20 pH 7.5, then MilliQ H_2O. Reaction product was developed with 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate kit (Zymed # 00-2014), and sections counterstained in hematoxylin for 15 seconds. All wash steps were in TBS/0.05% Tween-20. For the activin βB slides, the sections were washed and then incubated with Dako Envision HRP (rabbit, #K4003) for 1 hour at room temperature. The sections were washed again in TBS/Tween and the reaction product was developed with DAB, followed by counterstaining as for activin βA. Negative control sections were incubated with purified mouse myeloma IgG2B protein (Zymed #02-6300) instead of the activin βA-specific antibody or non-immunized rabbit IgG (Dako #X0903) instead of the activin βB-specific antibody.

Data analysis

For each sample, activin βA and βB mRNA expression levels were expressed relative to the GAPDH expression level for that sample. Thereafter, all time 0 data were normalized to 1 and data at subsequent time points was expressed relative to that time point. All data

are depicted as mean ± SEM values. Values were typically derived from the 3 tissue samples assessed per time point, but more samples were assessed in controls and at some early time points.

Results

In a mouse model of acute systemic inflammation following challenge with LPS, liver mRNA levels for the activin βA and βB subunits were examined. Activin βA subunit mRNA showed a minor increase (<2-fold control levels) in expression level 1 hour after LPS, but between 1 and 3 hours expression levels fell markedly and from 3 to 8 hours a clear suppression (to <25% of control levels) was evident (Figure 1, upper panel). By 12 hours, expression was approaching control levels and by 24 hours had returned to pretreatment levels. Treatment with the activin binding protein and antagonist, follistatin, resulted in an immediate suppression in activin βA subunit mRNA levels.

In contrast, liver βB subunit mRNA levels displayed a completely different profile to the activin βA subunit, rising immediately after LPS to reach a maximal expression level at 5 hours, at which time, expression averaged over 35-fold control levels (Figure 1, lower panel). Between 5 and 12 hours this expression fell progressively but at 12 hours, expression was still elevated (on average, 7-fold control levels). At 24 hours after LPS treatment, activin βB mRNA levels were still ~5- fold above control levels. As for activin βA subunit expression, activin βB subunit expression patterns were altered by the follistatin pretreatment, with clear suppression of the LPS-associated effects on βB subunit expression.

In the acute hepatic inflammation model following challenge with CCl_4, activin βA subunit expression fell slightly following CCl_4 treatment (Figure 2, upper panel), such that at 1 and 2 hours, average expression levels were only 40-50% of control levels. In contrast, by 4 hours after injection, average βA mRNA was moderately (80%) elevated and then declined to around pre-treatment levels by 36 hours. In contrast to the activin βA subunit, activin βB

showed the greatest changes in expression at 24 and 36 hours after CCl_4 injection, with a 13.5-fold increase above control levels (Figure 2, lower panel).

In both inflammatory models, expression of the inhibin α-subunit was examined but expression levels were consistently too low to permit quantitative analysis. Therefore it is unlikely that the profound changes in activin βB subunit mRNA resulted in the formation of elevated inhibin dimers (an α-βB dimer or inhibin B), but dimerized to form activin B (a dimer of βB-βB). Given the only marginal changes in activin βA mRNA, it is relatively unlikely that the increased βB mRNA expression resulted in significant formation of the heterodimer, activin AB (βA-βB).

Using antibodies specific for the activin βA and βB subunits, the liver immunolocalization was investigated in both the acute systemic model of LPS challenge and the acute hepatic inflammatory model using CCl_4. Localization of the activin βA subunit in normal liver was in hepatocytes and more specifically those predominantly around the central veins (Figure 3). Following LPS challenge, the localization appeared to diminish around 5 hours after LPS and returned to a pre-treatment distribution by 12 hours. For the activin βB subunit, however, localization was most evident in hepatocytes surrounding the portal tract areas of the liver and less so around the central veins (Figure 4). However, the localization appeared to diminish at 5 hours following LPS and returned to pre-treatment patterns by 12 hours. There also appeared to be a loss of hepatocyte localization in peripheral areas of the liver (Figure 4). In the CCl_4 model of acute hepatic inflammation, subunits localized to the hepatocytes surrounding the central vein and portal tracts for the βA and βB subunits respectively (Figure 5). However, 36 hours after CCl_4 treatment, there appeared to be localization for the activin βA subunit in hepatocytes that were destined to become apoptotic/necrotic, whereas there was no or little localization for the activin βB subunit in these areas (Figure 5).

EXAMPLE 2

INVESTIGATING WHETHER INFLAMMATION ALTERS CIRCULATING AMOUNTS OF ACTIVIN β_B DETECTED BY WESTERN BLOT

Detection of the activin β_B subunit was assessed using a specific polyclonal antibody that detects the activin β_B subunit. Sera from two experimental models of inflammation were tested. For the systemic inflammation model, male C57/BL6 mice were injected intraperitoneally (i.p.) with 100 μg phenol-purified LPS (*E. coli* 0127:B8, Sigma). For the acute hepatic inflammation model, male C57/BL6 mice were injected i.p. with carbon tetrachloride (CCl_4, 750 μl CCl_4/kg bodyweight in peanut oil).

Mouse serum from CCl_4- and LPS-treated mice at zero and five hours (LPS) and thirty-six hours (CCl_4) was diluted one in five in Radioimmunoprecipitation buffer (50mM Tris-HCl, 150mM NaCl, 1% Triton X-100, 0.25% Na-deoxycholate, 1mM EDTA) and boiled for 10 minutes in 2x reducing loading buffer (containing β-mercaptoethanol) as described previously by Laemmli (*Nature*, 1970, 227:680-685). Samples were run on a 12.5% running gel with a 4% stacking gel, alongside a prestained ladder (Fermentas PageRuler prestained protein ladder Cat# SM0671) and activin-β_B conditioned media from 293 cells. After electrophoresis for one hour at 130 volts, proteins were transferred to a PVDF membrane (Millipore Imobilon-FL, 0.45 μm pore size) overnight at 30 volts and 4 C, using the Biorad Mini-PROTEAN 3 apparatus. The membrane was blocked in Odyssey buffer (Li-Cor Biosciences) diluted one in four in PBS for one hour at room temperature (RT). A rabbit polyclonal antibody, developed by the Salk Institute and specific for the activin β_B subunit, was utilized (Vaughan *et al.*, 1989). The antibody, at 0.5 μg/ml, was added to the membrane for three hours at RT. Membranes were washed in 1xPBS/0.1% Tween, then incubated in Alexa Fluor goat anti- rabbit IgG (H+L) 680 secondary antibody, diluted 1:10,000 for one hour at RT in the dark. Membranes were washed again then visualized on an Odyssey Infrared Imaging System scanner.

The results of the Western blot (Figure 6) indicate that under reducing conditions, a specific band consistent with the activin β_B subunit is present at ~15 kDa, the expected size

of this subunit. This is confirmed in Lane 1 in a sample of conditioned medium from 293 cells overexpressing this subunit. Lanes 2 and 3 are from mice not challenged with inflammatory agents (LPS or CCl_4) and a band of the expected size is not apparent. Conversely, bands of the correct size were noted in sera from animals challenged with either LPS (Lanes 4 and 5) or CCl_4 (Lane 6). These findings are consistent with the activin β_B subunit, most probably activin B, being upregulated and released into the circulation following either systemic or local inflammation.

EXAMPLE 3

CHANGES IN THE β_B ACTIVIN SUBUNIT EXPRESSION, DETECTED BY IMMUNOCYTOCHEMISTRY IN TISSUE FROM PATIENTS AFTER A BURNS INJURY

Localisation of the activin β_B subunit was detected in tissue samples from burns injury patients using an immunohistochemistry technique and an antibody specific for this activin subunit. The immunohistochemistry protocol details are detailed in Example 1.

The expression of the β_B subunit was predominantly in the stratum germinativum and stratum spinosum, with the stratum granulosum and stratum lucidum being largely unlabelled (Figures 7A and B). In some instances, the expression of the β_B subunit was identified in the basal layers of the root of hair follicles (Figure 7C) and also in the basal layers of sebaceous glands (Figure 7D). Within the dermis, a mild localisation was also evident in secretory elements of sweat glands (Figure 7E) but the predominant localisation in the dermis was due to the presence of monocytes, neutrophils and lymphocytic infiltration (Figure 7F). These cellular elements often form large collections. In some areas, fibroblasts showed an easily detectable expression of the β_B subunit. The other major localisation of the β_B subunit was in the endothelium of blood vessels predominantly in capillaries that were present in the dermal layers (Figures 7F and G). Occasional profiles of arterioles showed some expression of the β_B subunit in the smooth muscle layers.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS:

1. A method for detecting the onset or a predisposition to the onset of an inflammatory response in a mammal, said method comprising screening for the level of activin protein, which activin comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression is indicative of an inflammatory response.

2. The method according to claim 1 wherein said activin is activin B.

3. The method according to claim 2 wherein said inflammatory response is a local inflammatory response.

4. The method according to claim 2 wherein said inflammatory response is a systemic inflammatory response.

5. The method according to claim 3 or 4 wherein said inflammatory response is related to septic shock, toxic shock, sepsis, septicaemia, pancreatitis, appendicitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, myocardial infarction, wound healing, traumatic injury, surgery or burns.

6. The method according to claim 5 wherein said inflammatory response is acute.

7. The method according to claim 4 wherein said systemic inflammatory response is related to systemic inflammatory response syndrome such as sepsis, septic shock, toxic shock, septicaemia, tissue trauma, meningitis or appendicitis.

8. The method according to claim 7 wherein said inflammatory response is acute.

9. A method for monitoring the progression of an inflammatory response in a

mammal, said method comprising screening for modulation of the level of activin protein, which activin comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression relative to a previously obtained level is indicative of the maintenance or worsening of said response and a decrease in said level is indicative of an improvement in said inflammatory response.

10. The method according to claim 9 wherein said activin is activin B.

11. The method according to claim 10 wherein said inflammatory response is a local inflammatory response.

12. The method according to claim 10 wherein said inflammatory response is a systemic inflammatory response.

13. The method according to claim 11 or 12 wherein said inflammatory response is related to septic shock, toxic shock, sepsis, septicaemia, appendicitis, pancreatitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, myocardial infarction, wound healing, traumatic injury, surgery or burns.

14. The method according to claim 13 wherein said inflammatory response is acute.

15. The method according to claim 12 wherein said systemic inflammatory response is related to systemic inflammatory response syndrome such as sepsis, septicaemia, tissue trauma, meningitis or appendicitis.

16. The method according to claim 15 wherein said inflammatory response is acute.

17. The method according to any one of claims 6, 8 or 16 wherein said level of activin B protein is at least 5 times higher than levels within the normal range.

18. The method according to claim 17 wherein said level of activin B protein is at least 7 times higher than levels within the normal range.

19. The method according to claim 18 wherein said level of activin B protein is at least 35 times higher than levels within the normal range.

20. A method for detecting the onset or a predisposition to the onset of a condition characterised by an inflammatory response in a mammal, said method comprising screening for the level of activin protein, which activin comprises a β_B subunit, and/or gene expression in said mammal where an increase in the level of said protein and/or gene expression is indicative of the onset or predisposition to the onset of said condition.

21. A method for monitoring the progression of a condition characterised by an inflammatory response in a mammal, said method comprising screening for modulation of the level of activin protein, which activin comprises a β_B subunit, and/or gene expression in said mammal wherein an increase in the level of said protein and/or gene expression relative to a previously obtained level is indicative of the maintenance or worsening of said condition and a decrease in said level is indicative of an improvement in said condition.

22. The method according to claim 21 wherein said activin is activin B.

23. The method according to claim 22 wherein said inflammatory response is a local inflammatory response.

24. The method according to claim 22 wherein said inflammatory response is a systemic inflammatory response.

25. The method according to claim 23 or 24 wherein said condition is septic shock,

sepsis, toxic shock, septicaemia, appendicitis, pancreatitis, meningitis, hepatic response to toxins or viruses, angiogenesis, psoriasis, neural protection, atherosclerosis, renal tubular necrosis, myocardial infarction, wound healing, traumatic injury, surgery or burns.

26. The method according to claim 25 wherein said inflammatory response is acute.

27. The method according to claim 24 wherein said condition is systemic inflammatory distress syndrome such as sepsis, septic shock, toxic shock, septicaemia, tissue trauma, meningitis or appendicitis.

28. The method according to claim 27 wherein said inflammatory response is acute.

29. The method according to claim 28 wherein the level of activin B protein is at least 5 times higher than levels within the normal range.

30. The method according to claim 29 wherein said level is at least 7 times higher than levels within the normal range.

31. The method according to claim 30 wherein said level is at least 35 times higher than levels within the normal range.

32. The method according to any one of claims 1-31 wherein said screening is directed to activin B.

33. The method according to any one of claims 4, 7, 8, 12, 15, 16, 24, 27 or 28 wherein said systemic inflammatory response is assessed based on analysis of peripheral levels of activin B.

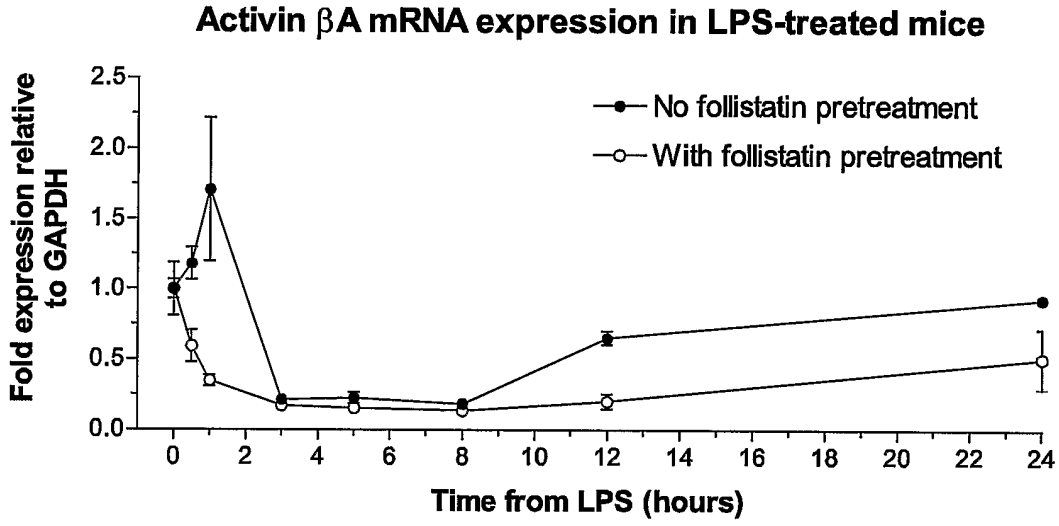
34. The method according to claim 33 wherein said peripheral levels of activin B are blood levels or CNS fluid levels.

35. The method according to claim 34 wherein said blood levels are assessed based on the analysis of a sample of blood or component derived therefrom.

36. The method according to claim 35 wherein said blood component is serum.

37. The method according to any one of claims 1 to 36 wherein said mammal is a human.

Figure 1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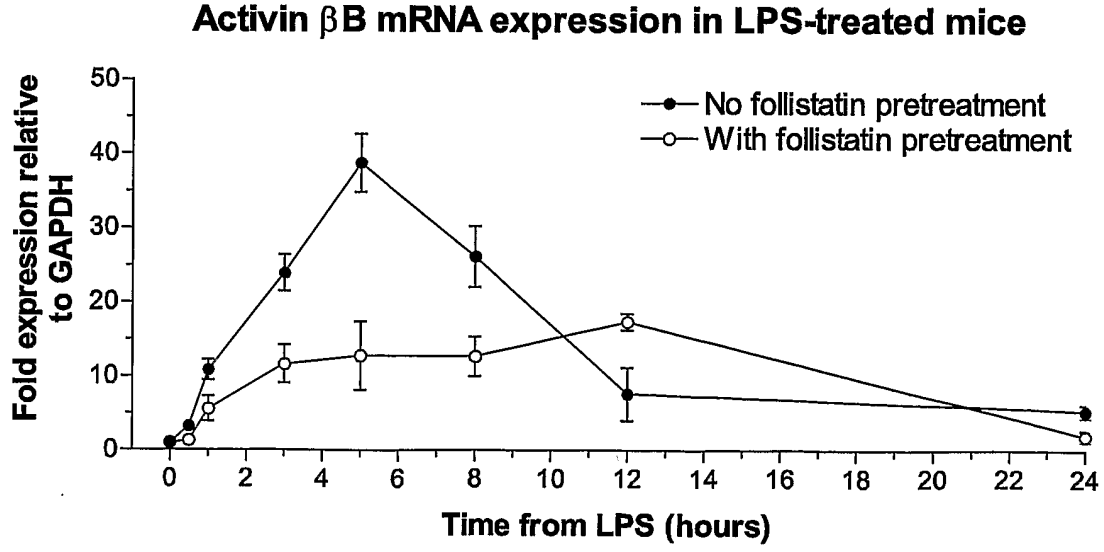
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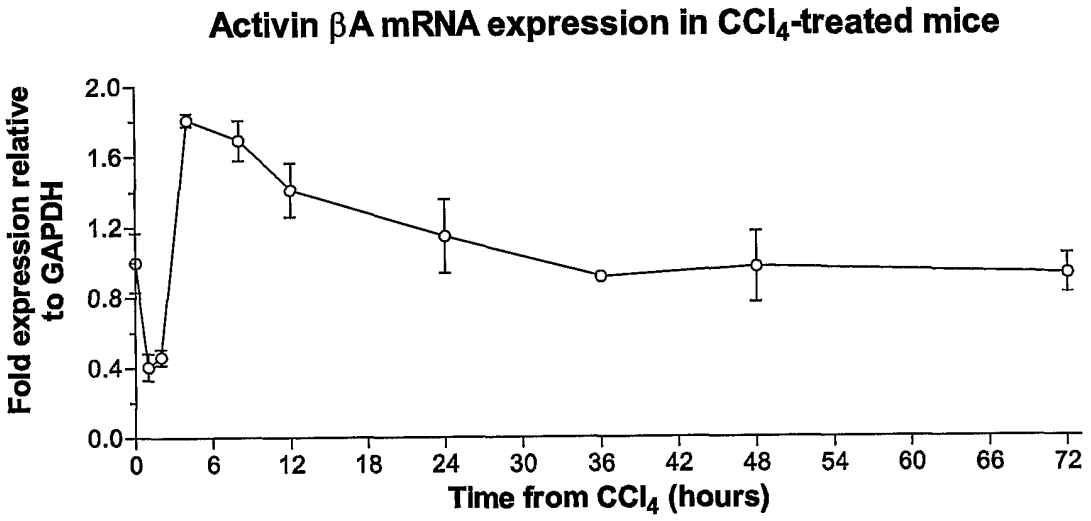
Time from LPS (hours)

Figure 2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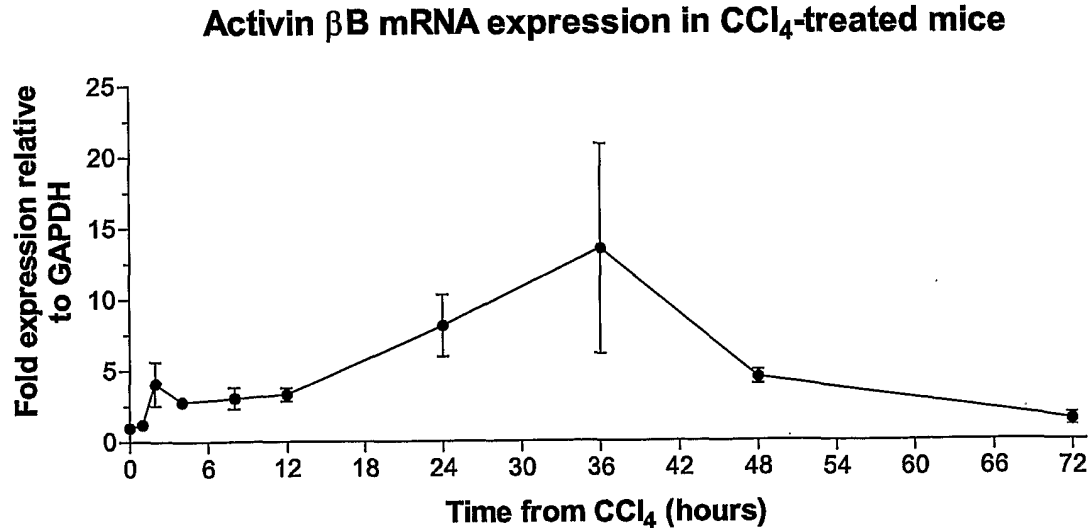
Time from CCl4 (hours)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Fold expression relative to GAPDH
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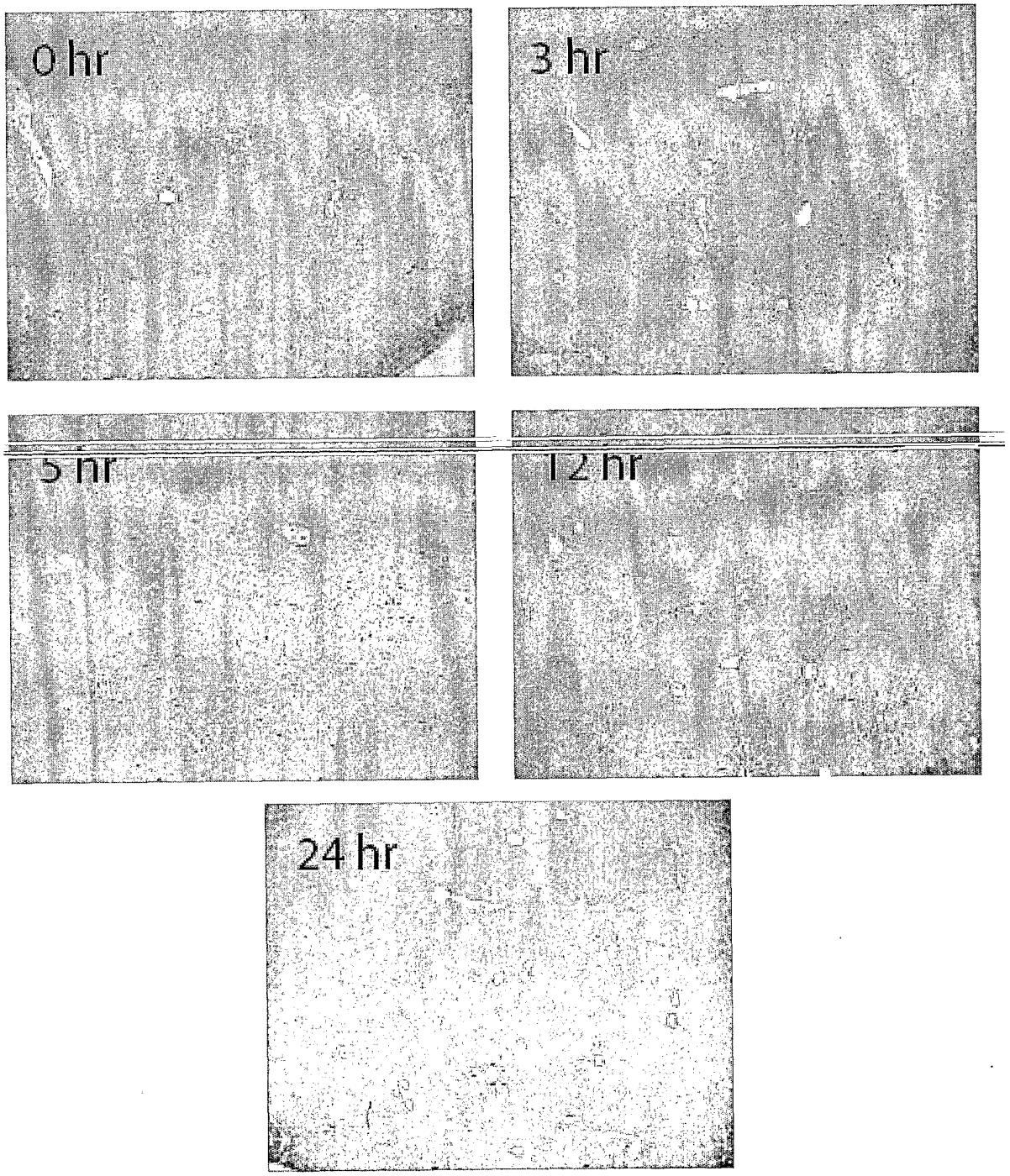
Time from CCl4 (hours)

Figure 3



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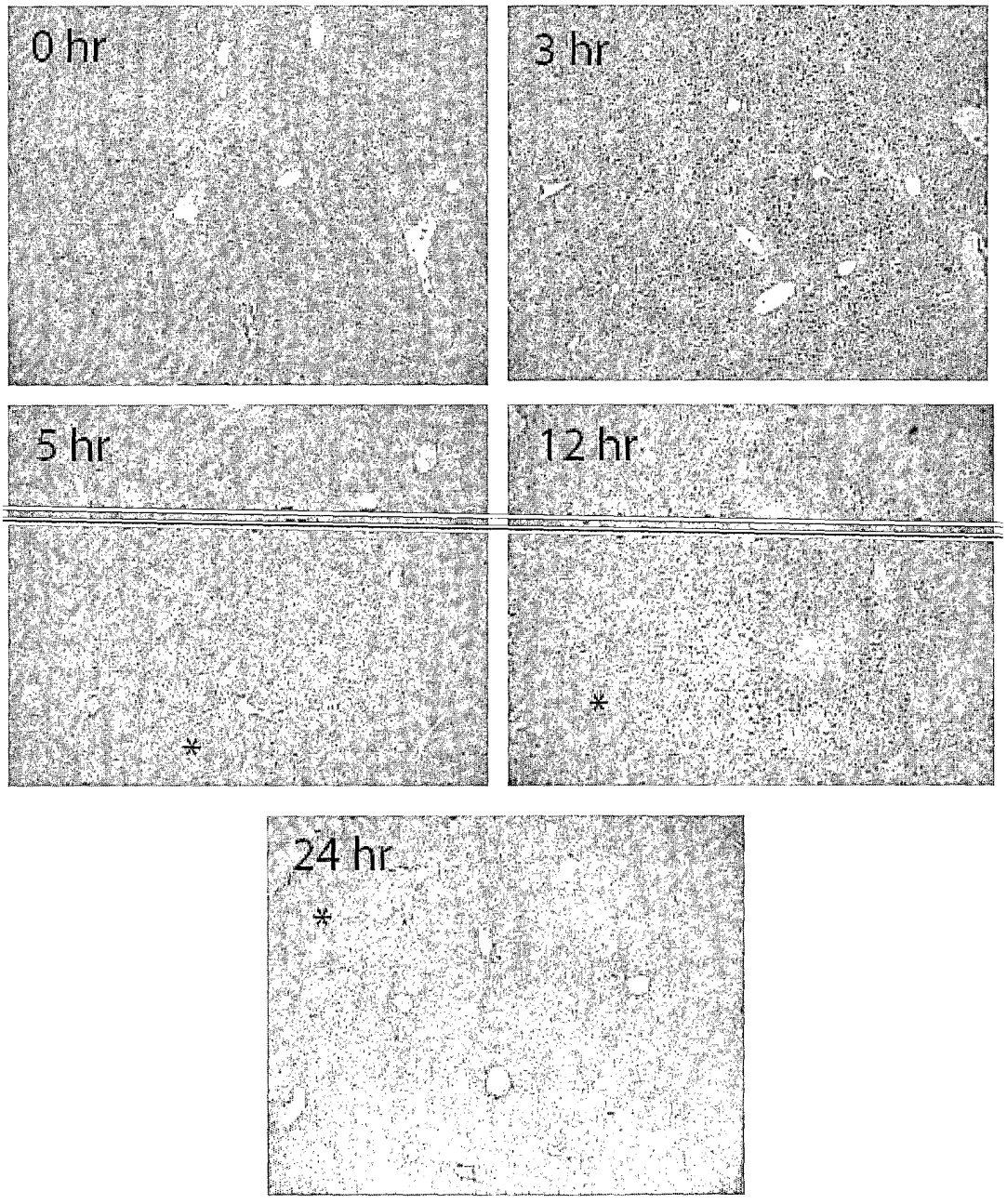

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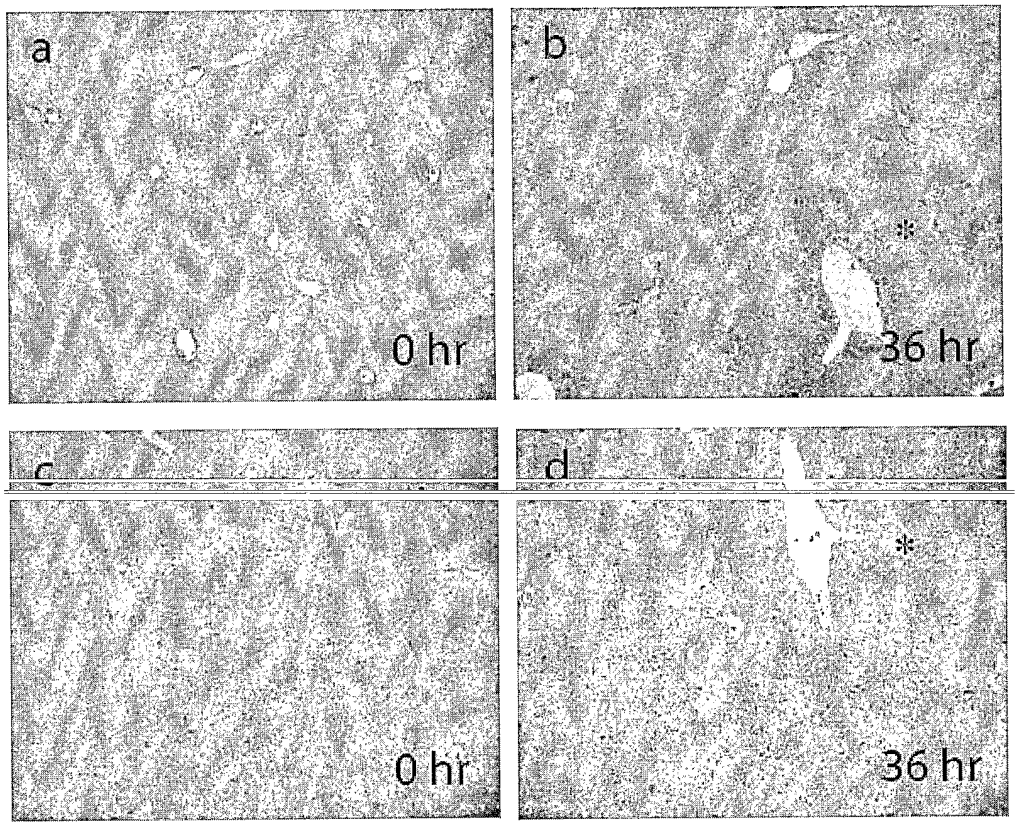

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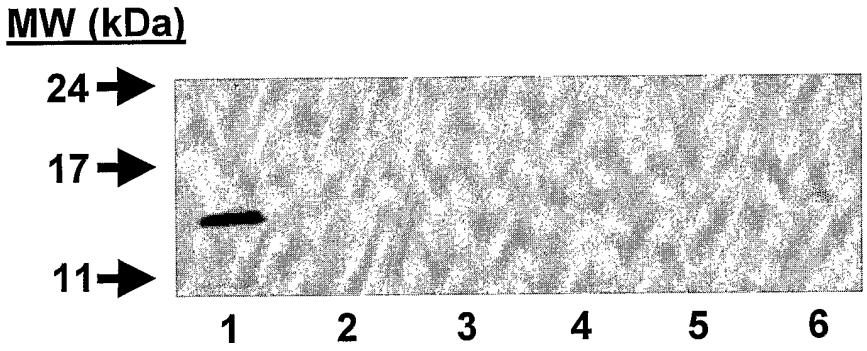

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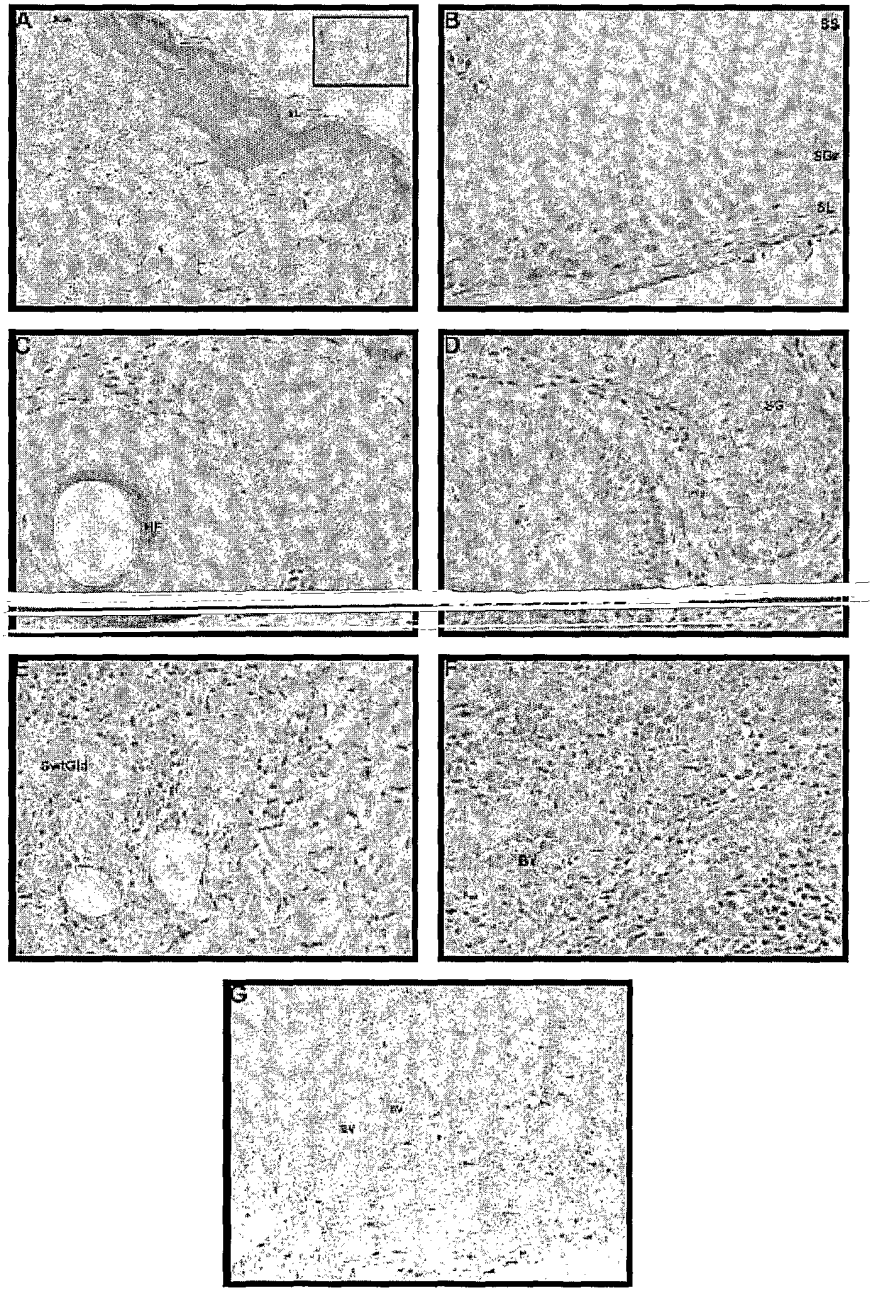

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2005/001270

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: C12Q 1/68

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)

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WPIDS, MEDLINE, CAPLUS,BIOSIS: activin β_B, activin B, activin AB, inflammation, inflammatory response, sepsis, septic

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 2005/032578 A1 (MONASH UNIVERSITY) 14 April 2005 See in particular page 8, lines 25-27; page 9, lines 15-23; examples 7 & 10; figures 6 & 7	1-37
A	WO 2004/053487 A1 (MONASH UNIVERSITY) 24 June 2004 See the whole document	1-37

☐ Further documents are listed in the continuation of Box C ☒ See patent family annex

* Special categories of cited documents:
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 October 2005	25 NOV 2005
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer **LEXIE PRESS** Telephone No : (02) 6283 2677

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/AU2005/001270

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Patent Document Cited in Search Report	Patent Family Member					
WO 2004053487	AU	2003287765	CA	2509539	EP	1579213
WO 2005032578						

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

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