

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
23 February 2006 (23.02.2006)

PCT

(10) International Publication Number  
**WO 2006/019203 A1**

(51) International Patent Classification<sup>7</sup>: **A61K 31/415**,  
A61P 9/00

(21) International Application Number:  
PCT/KR2004/002320

(22) International Filing Date:  
14 September 2004 (14.09.2004)

(25) Filing Language: Korean

(26) Publication Language: English

(30) Priority Data:  
10-2004-0064369 16 August 2004 (16.08.2004) KR

(71) Applicants (for all designated States except US): **SEOUL NATIONAL UNIVERSITY INDUSTRY FOUNDATION** [KR/KR]; San 4-2, Bongchun-dong, Gwanak-gu, Seoul 151-818 (KR). **STENTECH. INC.** [KR/KR]; #B104 Gae-poong Bld., 656-766, Sungsoo 1-Ga, Sungdong-gu, Seoul 133-110 (KR).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **KIM, Hyo-Soo** [KR/KR]; #401, Daewoomemberscounty 5-cha, 111-18, Cheongdam-dong, Gangnam-gu, Seoul 135-100 (KR).

**KOO, Bon-Kwon** [KR/KR]; 102-403, Hanwha Apt., Hongje-dong, Seodaemun-gu, Seoul 120-090 (KR). **KANG, Hyun-Jae** [KR/KR]; 219-701, Hangaram Apt., Ichon-dong, Yongsan-gu, Seoul 140-030 (KR). **YANG, Han-Mo** [KR/KR]; #402, 93-3, Yeongeon-dong, Jongno-gu, Seoul 110-460 (KR). **KWON, Byung-Doo** [KR/KR]; 102-702, Hyundaihangang Apt., 400, Ichon-dong, Yongsan-gu, Seoul 140-030 (KR).

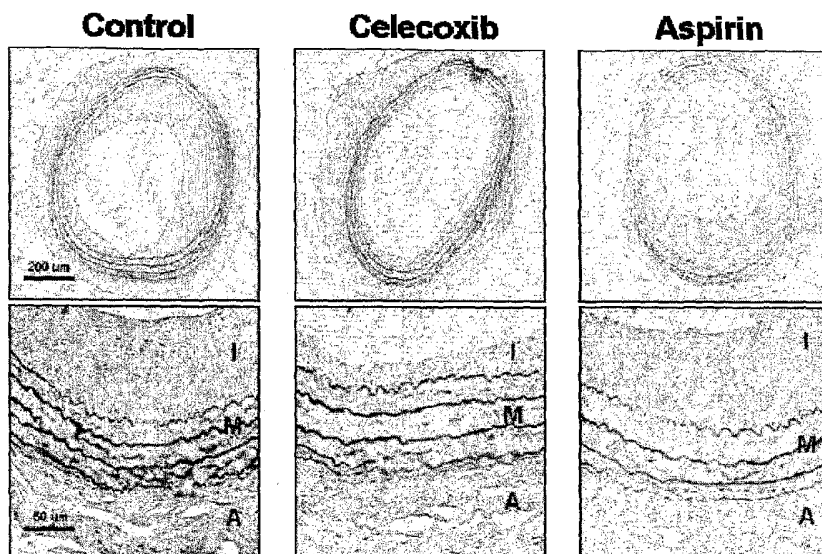
(74) Agent: **LEE, Keon-Cheol**; Keon Patent & Law Office, Sunggok Bldg., 4F, 823-22, Yeoksam-dong, Gangnam-gu, Seoul 135-080 (KR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

[Continued on next page]

(54) Title: PHARMACEUTICAL COMPOSITION FOR PREVENTING AND TREATING ARTERY RESTENOSIS CONTAINING CELECOXIB



(57) Abstract: This invention is directed to the use of certain pharmaceutical compositions containing celecoxib for preventing and treating vascular restenosis. The pharmaceutical compounds including celecoxib of the present invention can significantly reduce neointimal hyperplasia through an inhibition of VSMC (vascular smooth muscle cell) proliferation and an increase in VSMC apoptosis. From these results, celecoxib can be used for preventing and treating artery restenosis.

WO 2006/019203 A1



European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

**Published:**

— *with international search report*

**PHARMACEUTICAL COMPOSITION FOR PREVENTING AND TREATING**  
**ARTERY RESTENOSIS CONTAINING CELECOXIB**

**Technical Field**

This invention is directed to the use of certain pharmaceutical compositions containing celecoxib for preventing and treating vascular restenosis.

**Background Art**

Coronary artery disease has rapidly increased and is a major cause of morbidity and mortality in the world. Coronary intervention such as balloon angioplasty and stent implantation has been widely used to treat severe, symptomatic coronary stenosis. Although initially successful, an angioplasty procedure can be complicated by restenosis in 20-30% of procedures. The economic impact of treating restenosis is very high. As a result, numerous therapies to prevent restenosis have been tried. Until recently, drug-eluting stents using antiproliferative drugs are the most promising methods in preventing restenosis. Here, we focused our attention on celecoxib which is known to be safe in treating patients and has anti-proliferative and anti-tumor effects.

Celecoxib is a selective COX-2 inhibitor that has been used

extensively to safely treat patients with arthritis, and to have less gastrointestinal toxicity than NSAID (nonsteroidal anti-inflammatory drug)s (Bombardier C, Laine L, Reicin A, et al., *N Engl J Med.* 2000;343:1520-8, Silverstein FE, Faich G, Goldstein JL, et al., *J Am Med Assoc.* 2000;284:1247-55, FitzGerald GA, Patrono C., *N Engl J Med.* 2001;345:433-42). Moreover, treatment with celecoxib was found to reduce the number and size of colorectal polyps in patients with familial adenomatous polyposis (FAP) (Steinbach G, Lynch PM, Phillips RK, et al., *N Engl J Med.* 2000;342:1946-5). Based on these results, celecoxib has been approved as an adjunctive therapy in patients with FAP. Selective inhibitors of COX-2 have also been demonstrated to exert anti-proliferative effects and to induce apoptosis in colon, stomach, and prostate cancer cells (Hara A, Yoshimi N, Niwa M, et al., *Jpn J Cancer Res.* 1997;88:600-4, Erickson BA, Longo WE, Panesar N, et al., *J Surg Res.* 1999;81:101-7, Liu XH, Yao S, Kirschenbaum A, et al., *Cancer Res.* 1998;58:4245-9, Hsu AL, Ching TT, Wang DS, et al., *J Biol Chem.* 2000;275:11397-40), and clinical trials have recently shown that celecoxib has anti-tumor effects in lung, colon, and pancreatic cancer (Rahme E, Barkun AN, Toubouti Y, et al., *Gastroenterology.* 2003;125:404-12, Natale RB., *Oncology (Huntingt).* 2003;17:22-6, Crane CH, Mason K, Janjan NA, et al., *Am J Clin Oncol.* 2003;26:S81-4).

Recent evidence suggests that these anti-tumor and anti-proliferative effects of celecoxib are exerted through the

COX-independent pathways. In particular, it has been demonstrated that celecoxib induces apoptosis by blocking Akt (PKB, protein kinase B) activation(HsuAL, Ching TT, Wang DS, et al., *J Biol Chem.* 2000;275:11397-40) or by inhibiting 3-phosphositide-dependent kinase(PDK)-1 activity in cancer cells(Arico S, Pattingre S, Bauvy C, et al., *J Biol Chem.* 2002;277:27613-2). Akt regulates cellular processes including apoptosis, proliferation, migration and survival, all of which are involved in the development of neointimal hyperplasia. Akt activates both proliferative and anti-apoptotic signaling by stimulating growth factors such as VEGF, PDGF, and IGF(Brazil DP, Hemmings BA., *Trends Biochem Sci.* 2001;26:657-6). Akt has various downstream targets such as BAD, mTOR, and Forkhead family members(Brazil DP, Hemmings BA., *Trends Biochem Sci.* 2001;26:657-6). Akt also phosphorylates and inactivates GSK-3 $\beta$ . We and others have shown that the Akt-GSK axis is activated in the vessel wall after balloon injury and stent implantation(Park KW, Yang HM, Youn SW, et al., *Arterioscler Thromb Vasc Biol.* 2003;23:1364-9, Shigematsu K, Koyama H, Olson NE, et al., *Arterioscler Thromb Vasc Biol.* 2000;20:2373-8, Zhou RH, Lee TS, Tsou TC, et al., *Arterioscler Thromb Vasc Biol.* 2003;23:2015-2). Furthermore, We recently reported that constitutively active (the dephosphorylated form) GSK-3 $\beta$  gene transfer inhibits the proliferation of VSMCs, and reduces neointimal hyperplasia after balloon injury in the rats(Park KW, Yang HM, Youn SW, et al., *Arterioscler Thromb Vasc Biol.* 2003;23:1364-9).

Thus, we reasoned that celecoxib-mediated suppression of Akt might inhibit smooth muscle cell proliferation and neointimal hyperplasia in response to arterial injury after angioplasty or stent implantation. After performing these experiments from this idea, we confirmed that celecoxib has a significant effect as a drug for preventing and treating artery restenosis. Therefore, we could complete this invention from these results.

To investigate the effects of celecoxib on neointimal formation after balloon injury and its action mechanism, we examined whether celecoxib inhibits the proliferation of VSMCs by inhibiting PDGF-induced activation of Akt *in vitro*. We then examined whether celecoxib suppresses neointimal hyperplasia after balloon injury by blocking injury-induced activation of Akt *in vivo*. We also compared the effect of celecoxib and aspirin on neointima to investigate whether celecoxib exerts its effect through COX-dependent or COX-independent mechanism. In order to test the hypothesis that the Akt inhibition by celecoxib is the effector mechanism underlying the suppression of neointimal formation, we delivered adenoviral vectors expressing dominant-negative or constitutively active Akt genes to the rat carotid artery after balloon injury.

## **Disclosure of the Invention**

### **Technical Problem**

This invention is provided for the use of certain pharmaceutical compositions containing celecoxib for preventing and treating vascular restenosis.

### **Technical Solution**

To solve the above subject, this invention provides pharmaceutical composition for preventing and treating artery restenosis containing celecoxib.

The following part describes this invention in detail. From this invention, the pharmaceutical composition for preventing and treating artery restenosis contains celecoxib as a valid ingredient.

It is well known that Celecoxib in this invention has an effect on not only reducing inflammation but also reducing the number and size of colorectal polyps in patients with familial adenomatous polyposis (FAP). Moreover, celecoxib have also been demonstrated to exert anti-proliferative effects and to induce apoptosis in colon, stomach, and prostate cancer cells, and clinical trials have recently shown that celecoxib has anti-tumor effects in lung, colon, and pancreatic cancer.

### **Advantageous Effects**

These invention and experiments shows that celecoxib can significantly reduce neointimal hyperplasia through an inhibition of

VSMC(vascular smooth muscle cell) proliferation and an increase in VSMC apoptosis. From these results, celecoxib can be used for preventing and treating artery restenosis.

### **Brief Description of the Drawings**

Figure 1 depicts light microscopic findings of VSMCs treated with variable concentrations of celecoxib. (Magnification x200)

Figure 2 depicts Western Blot for phospho-Akt and phospho-GSK-3 $\beta$ , which shows that the stimulation of VSMCs with PDGF showed enhanced Akt phosphorylation compared to the untreated VSMCs. (t-Akt, total-Akt; p-Akt, phospho-Akt)

Figure 3 depicts WST-1 assay of VSMC viability after celecoxib treatment under PDGF(A) or 10% FBS(B) stimulation. \*  $P < 0.01$  vs vehicle-treated group. \*\*  $P < 0.01$  vs adeno-GFP at 25uM. Celecoxib 0uM, vehicle; myr-Akt, adenovirus of constitutively active Akt; GFP, adenovirus of Green Fluorescent Protein

Figure 4 depicts effects of celecoxib on DNA synthesis, the cell cycle, and apoptosis in PDGF culture condition.

(A) BrdU incorporation to measure DNA synthesis. (B) Cell cycle analysis (C) FACS analysis of the proportion of subdiploid cells.

\*  $P < 0.05$  vs vehicle (CXB 0uM) or only PDGF treatment. CXB, celecoxib.

Figure 5 depicts low and high magnification photomicrographs (elastic van Gieson staining) of the control, celecoxib, and the aspirin-treated groups. (I, Intima; M, Media; A, Adventitia)

Figure 6 depicts morphometric data.

(A) Intima to Media ratio (I/M ratio) (B) intimal, medial, luminal areas.

\*  $P < 0.01$  vs control group or aspirin treated group.

Figure 7 depicts the results of immunohistochemical staining.

(A) Immunohistochemical staining for PCNA at 3 days and 2 weeks. Black arrow head indicates PCNA-positive cell. (Magnification x400)

(B) TUNEL assay at 3 days and 2 weeks (first and second rows) and immunohistochemistry for activated caspase-3 (last row). . Black arrow head indicates TUNEL-positive cell. I, Intima; M, Media; A, Adventitia (Magnification x400)

Figure 8 depicts quantification of PCNA-positive and TUNEL-positive cell percentage in each group.

\*:  $P < 0.01$  vs control

Figure 9 depicts Western blot of arterial samples for phospho-Akt and phospho-GSK-3 $\beta$ .

(A) Natural course of phospho-Akt and phospho-GSK-3 $\beta$  after arterial injury.

(B) Western blot of arterial samples at 3 days after injury.

Figure 10 depicts effects of DN-Akt and myr-Akt gene transfer on in vivo neointima formation.

(A) Morphometric Data. I/M ratio, Intima to Media ratio; CXB, celecoxib; Myr-Akt, constitutively active Akt; DN-Akt, dominant negative-Akt; Con, control. \*  $P < 0.01$  vs control group.

(B) intimal, medial, luminal areas.

Figure 11 depicts immunohistochemical staining for PCNA and TUNEL assay. The black arrow head indicates PCNA-positive or TUNEL-positive cells.

(Magnification x400)

### **Best Mode**

In one embodiment of the present invention, it was implemented to evaluate the effects of celecoxib on VSMC viability and Akt/GSK-3 $\beta$  signaling pathway. As seen in figure 1, 2, and 3, celecoxib suppressed the phosphorylation of Akt and GSK in cultured VSMCs, which led to a reduction in viable cell number. This effect was reversed by transduction of constitutively active Akt. As seen in figure 4, the decrease in cell number after celecoxib treatment results from both decreased VSMC proliferation

and enhanced apoptosis.

In another embodiment of the invention, in vivo, it was implemented to compare the effects of celecoxib, aspirin, and vehicle on neointimal formation in rat carotid injury model. As seen in figure 5 and 6, celecoxib reduced Intima to Media ratio, whereas aspirin had no effect at 2 weeks post-injury. As seen in figure 7, 8, and 9, celecoxib reduced injury-induced phosphorylation of Akt and GSK, reduced VSMC proliferation, and increased caspase-3 activation and VSMC apoptosis at 3 days post-injury, whereas aspirin had no effect.

In yet another embodiment of the present invention, to support our hypothesis that the inhibition of Akt by celecoxib is the key mechanism underlying neointimal formation suppression, we delivered adenoviral vector expressing dominant-negative (DN)-Akt gene or active (myr)-Akt to rat carotid artery after balloon injury. As seen in figure 10 and 11, Adenovirus-mediated delivery of dominant-negative-Akt was as effective as celecoxib at inhibiting neointimal formation. Conversely, gene delivery of constitutively active Akt significantly reversed the inhibition of intimal hyperplasia by celecoxib, providing causal evidence that the modulation of Akt signaling by celecoxib is a physiologically relevant mechanism.

This celecoxib can be obtained commercially and used as unformulated form. Celebrex<sup>TM</sup> is a representative drug of celecoxib being sold at a market. In the present invention, celecoxib was purchased from Pfizer.

The pharmaceutical compositions of this invention can be selected

and adjusted in consideration of the state of patients, treatment duration and the purpose. For example, the concentration of celecoxib can be above 5uM. In the present invention, 5uM celecoxib can inhibit neointimal formation.

The pharmaceutical compositions of the present invention are very useful in preventing and treating vascular restenosis. Such vascular restenosis might be originated from neointimal hyperplasia. Moreover, this restenosis can be generated from the procedures such as vascular grafting including coronary artery bypass surgery, revascularization procedures including angioplasty, stent placement, endarterectomy, other invasive procedures involving arteries, veins and capillaries.

The pharmaceutical compositions of the present invention may include a pharmaceutically acceptable carrier or ingredient. Many pharmaceutically acceptable carriers may be employed. The suitable carriers include normal saline, buffered water, sterile water, glucose, sucrose, glycine, and glycerole. There is no limitation of acceptable carriers of the invention. The compositions may contain pharmaceutically acceptable auxiliary substances.

In particular embodiments of the invention, celecoxib is used, administered, or formulated with one or more second active ingredients to prevent and treat vascular restenosis. Ingredients and excipients employed in pharmaceutical compositions of the present invention include, but are not limited.

In another embodiments, the pharmaceutical compositions of the

present invention can be coated on a stent. The method of coating may include dip coating or polymer-based coating.

The compositions containing celecoxib can be in the form of, for example, a tablet, a pill, a hard or soft capsule, a lozenge, a cachet, a dispensable powder, granules, a suspension, an elixir, a liquid, or any other form reasonably adapted for oral or parenteral administration.

For an adult human, a therapeutically effective amount of celecoxib for preventing and treating vascular restenosis is about 50mg to about 800mg a day. The daily dosage and frequency of administration, and therefore the selection of appropriate dose unit, depends on a variety of factors, including the age, weight, sex and medical condition of the subject, and the nature and severity of the condition or disorder, and thus may vary widely.

#### **Mode of invention**

As is indicated herein below, there are some examples for the present invention. However, these are the simple example of the present invention and thus the whole contents of the present invention are not limited to these examples.

#### **EXAMPLES**

##### **Process for Making Pharmaceutical Compounds Including Celecoxib**

Purified celecoxib was purchased from Pfizer Corporation, and

dissolved in dimethyl sulfoxide (DMSO) and the final concentration of the vehicle in all cultures was 0.1%.

### **Process for Making Adenoviral Vectors**

To modulate Akt activity, we used two adenoviral constructs tagged with the HA(hemagglutinin) epitope, as described previously(Fujio Y, Walsh K., *J Biol Chem.* 1999;274:16349-54, Fujio Y, Guo K, Mano T, et al., *Mol Cell Biol.* 1999;19:5073-82). The DN(dominant-negative)-Akt construct contains two phosphorylation sites; serine 473 and threonine 308, both mutated to alanine, which results in an unphosphorylatable form of Akt. Myr-Akt is Akt which is myristoylated at its N-terminal end, resulting in its constitutively active form.

### **Experimental Animals**

Male Sprague-Dawley rats, 13 weeks old, weighing around 400g (Daehan Biolink Co) were fed with standard pellet feed and given water *ad libitum*. The experimental protocol was designed in accordance with the Guide for Experimental Animal Research issued by the Laboratory for Experimental Animal Research, Clinical Research Institute, Seoul National University Hospital.

### **Statistical Analysis**

Data are presented as mean  $\pm$  SEM. Analysis was performed by one-way ANOVA with Bonferroni's test for multiple comparisons, and by the Student's

t test using SPSS 11.0 software. A P value of < 0.05 was considered significant.

#### **EXAMPLE 1 : Effects of Celecoxib on Reducing VSMC Viability in vitro**

##### **1-1) Cell Isolation and Culture**

Rat aortic VSMCs were isolated from the thoracic aortas of Sprague-Dawley rats by enzymatic dispersion using a slight modification of a previously described method (Sachinidis A, Flesch M, Ko Y, et al., *Hypertension*. 1995;26:771-8). Cells were cultured in Dulbecco's Modified Eagle Media (DMEM, Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 IU/ml streptomycin. The purity of VSMC cultures was confirmed by the immunocytochemical detection of  $\alpha$ -smooth-muscle actin.

##### **1-2) Analysis of Effects of Celecoxib on VSMC viability and Akt phosphorylation by Western Blot**

Confluent cells cultured in 6 cm dishes were incubated in serum-deprived media for 24hrs. VSMCs were then stimulated using 25ng/ml of PDGF-BB in the presence of different celecoxib concentrations (0, 10, 25 mM) for 2hrs. After removing the medium, cells were lysed in buffer. Western blot analysis was performed as described previously.<sup>14</sup> The primary antibodies used were anti-phospho-Akt (Ser473) (1:500 dilution, Cell Signaling Technology), anti-total-Akt (1:500 dilution, Cell Signaling Technology), anti-phospho-GSK-3 $\beta$  (Ser9) (1:750 dilution, Cell Signaling

Technology) and anti- $\alpha$ -tubulin (1:4000 dilution, Oncogene). For *in vivo* studies, 3 carotid arteries were pooled in each group. Tissue samples were homogenized in sample buffer.

On microscopic examination, VSMCs treated with 10 or 25 $\mu$ M of celecoxib became rounded and detached from the culture dish (Figure 1).

The loss of cell viability was associated with a significant attenuation of both phosphorylated Akt and phosphorylated GSK-3 $\beta$ , a downstream substrate of Akt. Western blot analysis showed that celecoxib markedly reduced the basal level of phospho-Akt and phospho-GSK-3 $\beta$  in non-stimulated conditions (Figure 2). The enhanced Akt phosphorylation and paralleled increase in the phospho-GSK-3 $\beta$  after PDGF stimulation was also reduced in a dose-dependent manner with celecoxib treatment (Figure 2).

### **1-3) Analysis of Effects of Celecoxib on VSMC viability by WST-1 Assay**

The WST-1 assay was used to quantify the effect of celecoxib on the decrease in cell number and to examine whether its effect is mediated through the inhibition of Akt pathway.

Cell proliferation was measured using the WST-1 assay (Roche Molecular Biochemicals) according to the manufacturer's instructions. Cells were seeded in 96-well plates at  $2 \times 10^4$  cells per well in DMEM (100 $\mu$ L) supplemented with 10% FBS. After 24hrs, the medium was replaced by 10% FBS-DMEM containing various concentrations of celecoxib (0, 5, 10, 25 $\mu$ M) for 48hrs. At the end of the incubation period, 10 $\mu$ L of WST-1 was added for 2 hours. For *in vitro* gene transfer experiments, VSMCs were transfected with adenoviral vectors

(50 m.o.i) overnight before adding celecoxib.

This assay showed that celecoxib decreased the number of viable VSMCs in the presence of either PDGF or 10% FBS in a dose-dependent manner (Figure 3). The inhibitory effect of celecoxib on viable cell number was more potent with PDGF than with 10% FBS (viable cells,  $41.2 \pm 6.7$  % at celecoxib 10uM,  $27.3 \pm 4.4$  % at 25uM compared to 100% at vehicle under PDGF stimulation,  $76.8 \pm 7.0$  % at celecoxib 10uM,  $63.6 \pm 8.6$  % at 25uM compared to 100% at vehicle under 10% FBS stimulation, both  $p < 0.01$ ). Transduction of Akt with constitutively active adeno-myr Akt reversed the action of celecoxib resulting in a significant increase in viable cells ( $27.3 \pm 4.4$  % with adeno-GFP,  $91.3 \pm 13.2$  % with adeno-myrAkt under PDGF;  $63.6 \pm 8.6$  % with adeno-GFP,  $84.8 \pm 8.5$  % with adeno-myrAkt under 10% FBS at celecoxib 25uM, both  $p < 0.01$ ) (Figure 3).

#### **1-4) Analysis of Effects of Celecoxib on Cell cycle and Apoptosis by BrdU Incorporation Assay and Flow Cytometry**

##### **BrdU Assay**

This assay is quite similar to WST-1 assay except using BrdU instead of WST-1.

##### **Flow Cytometry**

Cell-cycle status and apoptosis were evaluated by flow cytometry, as described previously (Kim HS, Skurk C, Thomas SR, et., J Biol Chem. 2002;277:41888-9). Cells were plated, allowed to attach overnight, and placed in DMEM plus 0.4% FBS for 48hours. Celecoxib was then added in a

serum-free medium composed of DMEM with 25ng/mL PDGF-BB or 10% FBS. After 24 hours, the cells were harvested and fixed. DNA content was analyzed by flow cytometry (Becton-Dickinson). Histograms of DNA contents were analyzed using Modfit LT software (Verity Software, Topsham, ME) to characterize population fractions in each phase of the cell cycle.

### **Results**

Celecoxib inhibited DNA synthesis in a dose-dependent manner (Figure 4A). This treatment arrested the cell cycle at G1 phase resulting in a decrease in the fraction of cells in the S phase (Figure 4B). Celecoxib significantly increased the subdiploid fraction of DNA by FACS analysis which is indicative of DNA fragmentation during apoptosis (Figure 4C). The effects of celecoxib on BrdU incorporation, S phase fraction, and apoptosis were greater with PDGF culture conditions compared with 10% FBS (data not shown).

### **EXAMPLE 2 : Effects of Celecoxib on Reducing Neointimal Hyperplasia in vivo**

In the first *in vivo* study, rats were divided into three groups (n=10/group): celecoxib (50mg/kg/day), aspirin(50mg/kg/day), and vehicle (0.5% methylcellulose, 0.025% Tween 20, Sigma Chemical Co.) groups. Drugs were administered daily by oral gavage, in a 0.5 ml suspension of vehicle, for 3 days before the balloon injury, and were continued for two weeks after injury.

### 2-1) Process of Rat Carotid Artery Balloon Injury Model

Animals were anesthetized with ketamine hydrochloride (50mg/kg, Yuhan Corp, Bayer Korea) and xylazine (7mg/kg, Yuhan Corp, Bayer Korea). The left carotid artery was exposed, and a 2F Fogarty balloon embolectomy catheter (Baxter) was inserted via an external carotid arteriotomy incision (Clowes AW, Reidy MA, Clowes MM., *Lab Invest.* 1983;49:327-33). The catheter was advanced to the common carotid artery, inflated with 0.2mL of saline, and withdrawn 3 times with rotation.

### 2-2) Morphometric Analysis

At 3 days or 2 weeks after injury rats were euthanized by pentobarbital overdose and perfused with 10% formaldehyde. Carotid arteries were removed and placed in the same fixative. Tissues were then embedded in paraffin, and 4 to 5 sections (4um) were cut at multiple levels. These were stained with Hematoxylin & Eosin or elastic van Gieson stain. Sections were then examined and the cross sectional area of the lumen, neointimal area (from the internal elastic lamina to the lumen), and medial area were determined using an Image Pro Plus Analyzer Version 4.5 (Media Cybernetics Inc.). The intima to media (I/M) ratio was then calculated from the determined means.

At 2 weeks after injury, the vehicle-treated group showed abundant neointimal hyperplasia (Figure 5). The celecoxib-treated group showed significant suppression of neointimal hyperplasia and reduction of the I/M ratio (I/M ratio; Vehicle versus Celecoxib,  $0.98 \pm 0.04$  versus  $0.48 \pm 0.05$  mm<sup>2</sup>,  $P < 0.001$ ). In contrast, the aspirin-treated group showed no significant

reduction of neointimal hyperplasia (I/M ratio; Vehicle versus Aspirin,  $0.98 \pm 0.04$  versus  $0.89 \pm 0.05 \text{ mm}^2$ ,  $P = 0.25$ ) (Figure 6). At 3 days after injury, no intimal hyperplasia could be detected in the three groups (data not shown).

### **2-3) Analysis of Effects of Celecoxib on VSMC Proliferation and Apoptosis in vivo**

To examine the effects of celecoxib on VSMC proliferation *in vivo*, IHC for PCNA was performed. Moreover, to examine the effects of celecoxib on VSMC apoptosis *in vivo*, we performed TUNEL assay and IHC for activated caspase-3.

#### **Immunohistochemical Staining**

Immunohistochemistry(IHC) was performed as previously described (Park KW, Yang HM, Youn SW, et al., *Arterioscler Thromb Vasc Biol.* 2003;23:1364-9). The primary antibodies used were anti-PC10, for proliferating cell nuclear antigen (PCNA 1:200 DAKO), anti-HA (1:200 Santa Cruz), anti-cleaved caspase-3 (1:200 Cell Signaling Technology), and anti-ED1 for macrophages (1:200 Serotec).

#### **TUNEL Assay**

The TUNEL assay was performed with minor modification to a previously described method (Park KW, Yang HM, Youn SW, et al., *Arterioscler Thromb Vasc Biol.* 2003;23:1364-9), using an Apoptag kit (Intergen Company). DAB

was used as a chromogen. Sections were counterstained with methyl green or Mayer's hematoxylin. The percentage of PCNA-positive, TUNEL-positive and ED1-positive cells versus total nucleated cells was quantified in 3 different sectors per tissue section. All samples were coded so that analysis was performed without knowledge of which treatment each individual vessel had received.

#### **Effects of Celecoxib on Inhibiting VSMC Proliferation**

To examine the effects of celecoxib on VSMC proliferation *in vivo*, IHC for PCNA was performed. At 3 days,  $29.8 \pm 1.7\%$  of the nuclei in the vehicle-treated group were PCNA-positive, whereas only  $17.1 \pm 1.5\%$  in the celecoxib-treated group were PCNA-positive ( $P < 0.001$ ) (Figure 7A, 8A). At 2 weeks, the PCNA-positive percentage in the vehicle-treated group was  $9.7 \pm 0.8\%$ , whereas in the celecoxib-treated group it was  $5.9 \pm 0.8\%$ . However, no inhibition of proliferation was found in the aspirin-treated group (3 days;  $25.6 \pm 1.9\%$ ; 2 weeks,  $8.5 \pm 0.7\%$ ).

#### **Effects of Celecoxib on Inducing VSMC Apoptosis**

To examine the effects of celecoxib on VSMC apoptosis *in vivo*, we performed TUNEL assay and IHC for activated caspase-3. At 3 days after injury, the celecoxib-treated group showed higher levels of apoptosis than either the vehicle- or aspirin-treated groups (Celecoxib versus Vehicle versus Aspirin;  $19.4 \pm 2.0\%$  versus  $12.3 \pm 1.3\%$  versus  $11.8 \pm 1.2\%$ ). At 2 weeks, this tendency was also present, but to a lesser extent (Celecoxib versus Vehicle

versus Aspirin,  $8.1 \pm 0.9\%$  versus  $4.6 \pm 0.7\%$  versus  $5.3 \pm 0.8\%$ ). Consistent with these data, IHC for activated caspase-3 showed more staining in the celecoxib-treated group (Figure 7B, 8B).

In tissues harvested 2 weeks after injury, the location of macrophage in the neointima was not periluminal, but near the internal elastic lamina (data not shown). In contrast to the vehicle-treated group, the aspirin- and celecoxib-treated groups showed a small reduction in macrophage infiltration (macrophage percent, Control versus Aspirin versus Celecoxib,  $7.1 \pm 0.9\%$  versus  $5.1 \pm 0.6\%$  versus  $5.4 \pm 0.5\%$ ).

#### **2-4) Analysis of Effects of Celecoxib on Akt phosphorylation by Western Blot in vivo**

To examine the time course of Akt activation after injury *in vivo*, Western blotting for phospho-Akt and phospho-GSK-3 $\beta$  was performed with the tissues taken before injury, and 3 days and 2 weeks after injury. Akt activity at 3 days after injury was significantly elevated as was the phosphorylation of GSK-3 $\beta$  (Figure 9A). At 2 weeks after injury, activated Akt levels decreased dramatically. To investigate the mechanism of celecoxib action following vascular injury, Western blot analysis for Akt and GSK-3 $\beta$  was performed in all three groups at 3 days after balloon injury. At this time point, the levels of phosphorylated Akt and GSK-3 $\beta$  were significantly suppressed in the celecoxib-treated group compared to the vehicle- and aspirin-treated groups (Figure 9B).

**EXAMPLE 3 : Effects of Celecoxib on Reducing Neointimal Hyperplasia Through Inhibiting Akt Signaling in vivo**

In the second *in vivo* study, rats that underwent balloon denudation injury of the carotid artery were divided into four groups (n=10/group): Adeno-Green Fluorescent Protein (GFP), Adeno-DN-Akt, celecoxib + adeno-GFP, and celecoxib + constitutively active (myr)-Akt. After balloon injury,  $5 \times 10^8$  pfu of adenovirus diluted in a total volume of 20  $\mu$ L was delivered to injured segments, which was then incubated for 20min with vascular clamp.

**3-1) Analysis of Effects of DN-Akt (Inactive Form) Gene Transfer on Reducing Neointimal Hyperplasia**

To test whether the inhibition of Akt activation by celecoxib contributes to the suppression of neointimal hyperplasia, we evaluated the effect of dominant-negative Akt gene transfer in the balloon injury model. Expression of the HA-tagged transgene was detected by IHC at 2 weeks after delivery. HA-tagged transgene expression was also detected by Western immunoblot analysis (data not shown).

At 14 days after injury, DN-Akt gene transfer led to a significant reduction in intimal hyperplasia (I/M ratio; Control versus DN-Akt,  $0.86 \pm 0.05$  versus  $0.51 \pm 0.04 \text{ mm}^2$ ,  $P < 0.001$ ) (Figure 10A). A significant decrease in the percentage of PCNA-positive VSMCs was also observed in the neointima and media of DN-Akt group at 3 days and 2 weeks (Control versus DN-Akt; 3days:  $25.4 \pm 1.7\%$  versus  $15.7 \pm 1.8\%$ , 2weeks:  $7.6 \pm 0.9\%$  versus  $4.7 \pm 0.6\%$ ,  $P < 0.05$ ). At 3 days after injury, the DN-Akt-treated group showed more

apoptosis by TUNEL assay (Control versus DN-Akt;  $13.8 \pm 1.6\%$  versus  $19.7 \pm 2.1\%$ ,  $P < 0.05$ ), and at 2 weeks after injury, although the absolute value decreased, the tendency was sustained (Control versus DN-Akt;  $3.3 \pm 0.5\%$  versus  $5.3 \pm 0.7\%$ ,  $P < 0.05$ ) (Figure 11).

### **3-2) Analysis of Effects of Myr-Akt (Active Form) Gene Transfer on Neointimal Formation**

To test that modulation of Akt signaling by celecoxib is a physiologically relevant mechanism, we delivered adenoviral vectors expressing constitutively active Akt genes to the rat carotid artery after balloon injury. At 2 weeks after injury, myr-Akt gene transfer reversed the inhibition of intimal hyperplasia by celecoxib (I/M ratio; adeno-GFP versus celecoxib + adeno-GFP versus celecoxib + myr-Akt,  $0.86 \pm 0.05$  versus  $0.45 \pm 0.04$  versus  $0.78 \pm 0.05\text{mm}^2$ ) (Figure 10A). In IHC for PCNA, myr-Akt gene transfer reversed the inhibition of VSMC proliferation by celecoxib (celecoxib + adeno-GFP versus celecoxib + myr-Akt ; 3days:  $16.9 \pm 1.3\%$  versus  $20.7 \pm 1.3\%$ , 2weeks:  $5.1 \pm 0.5\%$  versus  $6.9 \pm 0.7\%$ ,  $P < 0.05$ ) (Figure 11). In the TUNEL assay, myr-Akt gene transfer reduced the level of apoptosis induced by celecoxib (celecoxib + adeno-GFP versus celecoxib + myr-Akt ; 3days:  $21.3 \pm 1.6\%$  versus  $15.4 \pm 1.4\%$ , 2weeks:  $6.1 \pm 0.6\%$  versus  $4.1 \pm 0.5\%$ ,  $P < 0.05$ ).

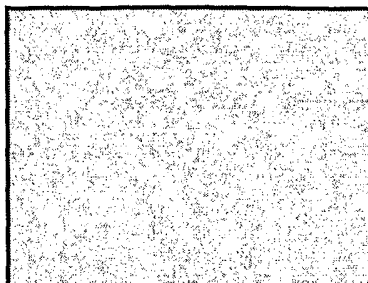
### Claims

1. Pharmaceutical compositions containing celecoxib as an active ingredient for the treatment and prevention of vascular stenosis.

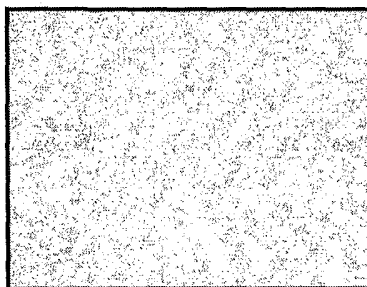
2. Pharmaceutical compositions according to claim 1, wherein vascular stenosis may be due to neointimal hyperplasia.

3. Pharmaceutical compositions according to claims 1 or 2, wherein vascular stenosis may be induced by percutaneous transluminal coronary angioplasty, atherectomy, stent implantation, coronary artery bypass grafting and arteriovenous anastomosis.

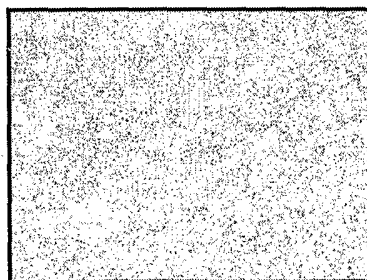
**FIG.1**



**Vehicle only**



**Celecoxib 10uM**



**Celecoxib 25uM**

FIG.2

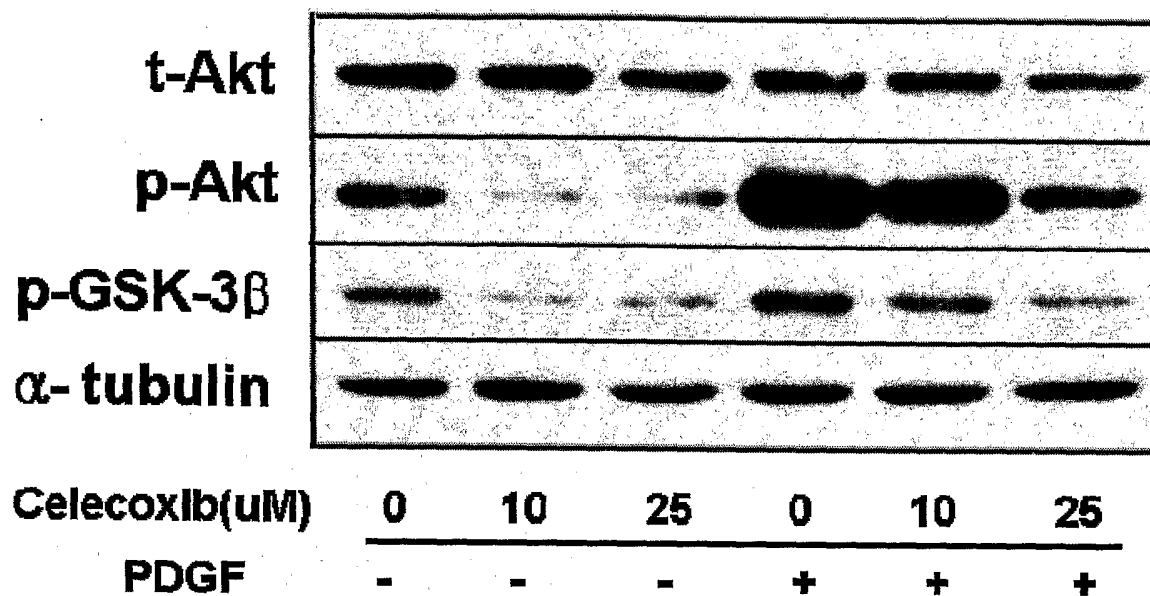
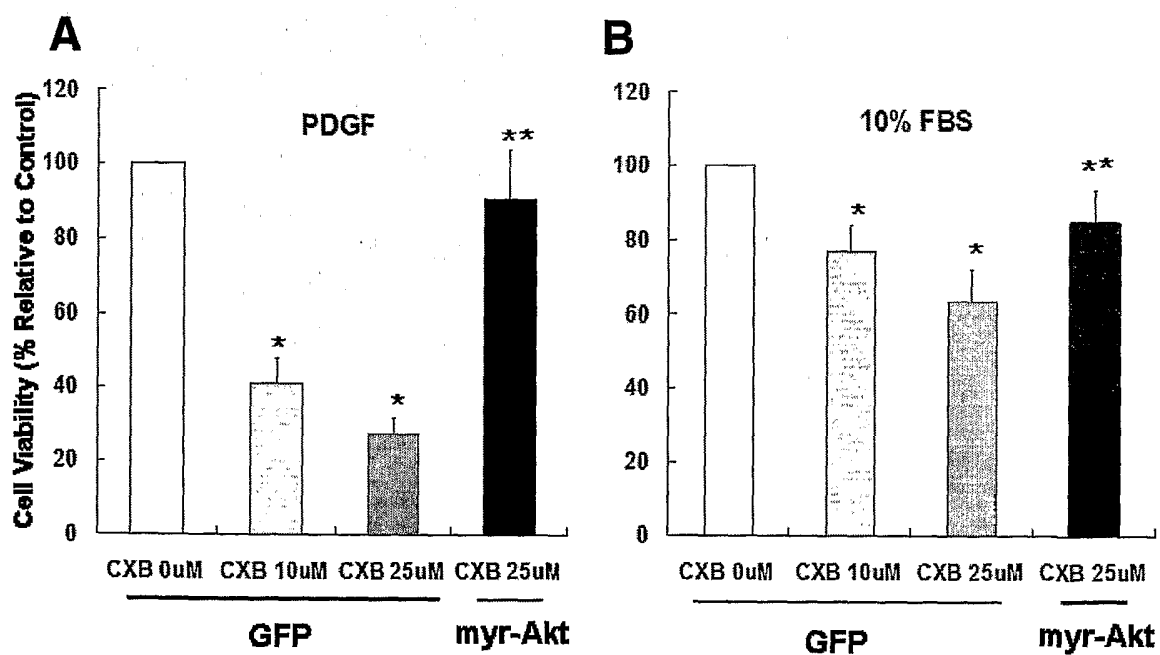


FIG.3

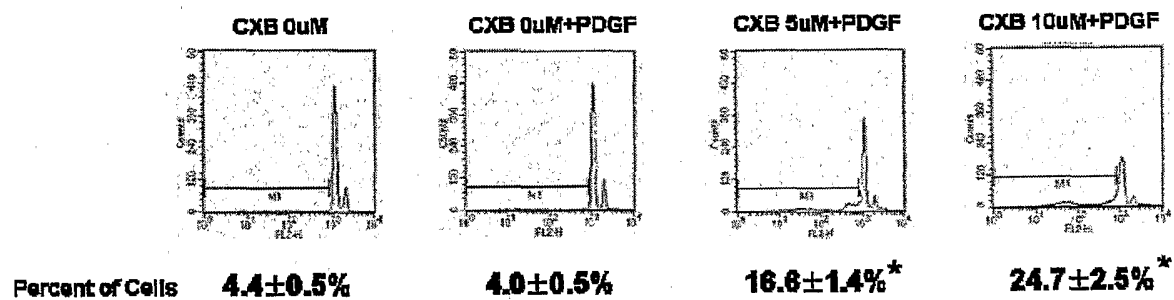


**FIG.4****A BrdU Incorporation**

|              | CXB 0uM  | CXB 0uM+PDGF | CXB 5uM+PDGF | CXB 10uM+PDGF |
|--------------|----------|--------------|--------------|---------------|
| % of Control | 81.2±7.4 | 100±7.5      | 68.1±9.5*    | 29.4±5.3*     |

**B Cell Cycle**

| % of Cells | CXB 0uM  | CXB 0uM+PDGF | CXB 5uM+PDGF | CXB 10uM+PDGF |
|------------|----------|--------------|--------------|---------------|
| G1         | 85.8±2.2 | 81.2±1.1     | 87.3±1.3*    | 92.7±2.1*     |
| S          | 6.8±1.4  | 10.3±1.2     | 5.3±1.4*     | 3.1±1.0*      |
| G2-M       | 7.5±0.9  | 8.5±0.2      | 7.5±1.2      | 4.2±1.4*      |

**C Apoptosis FACS**

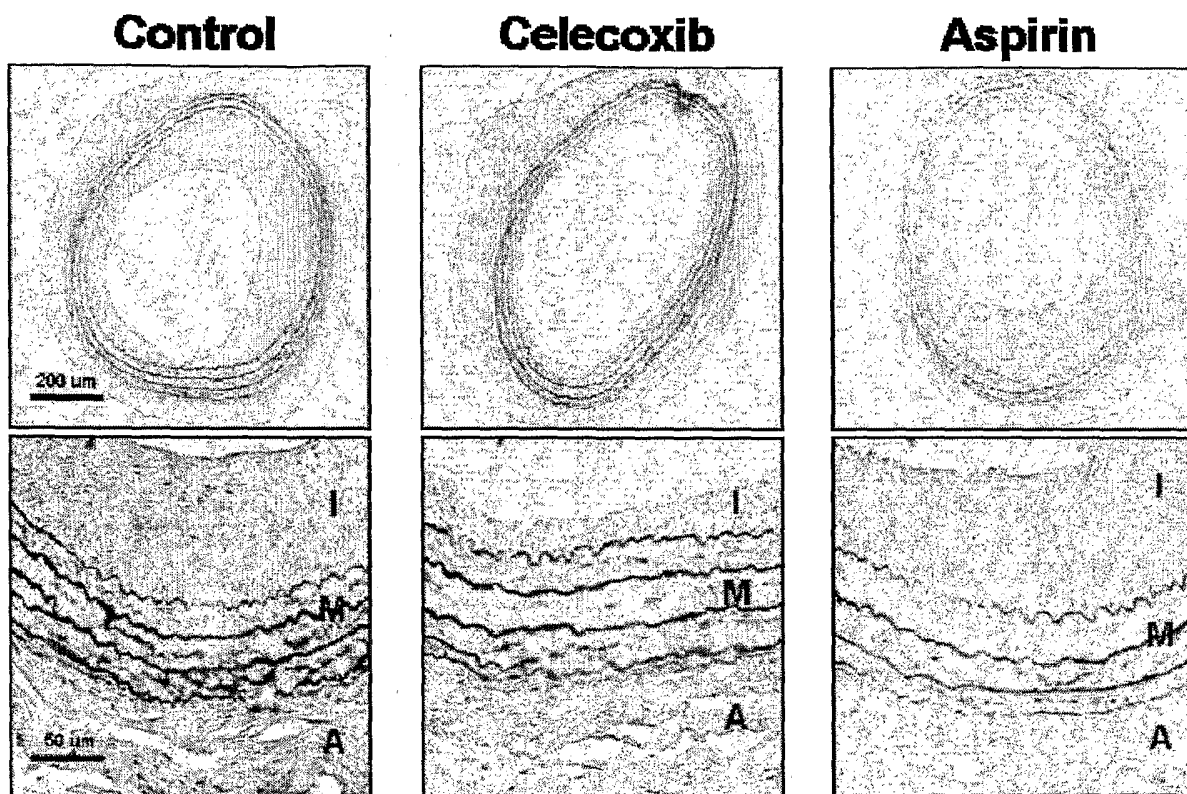
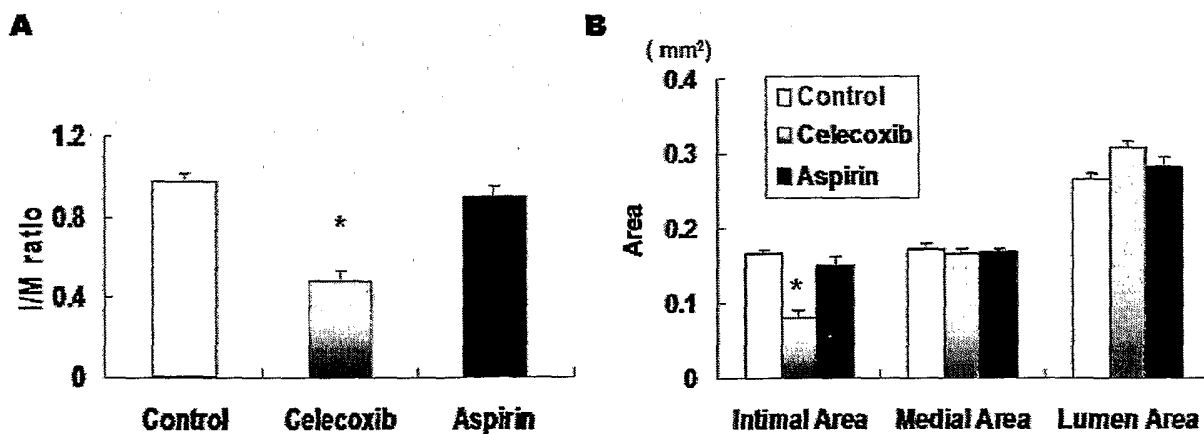
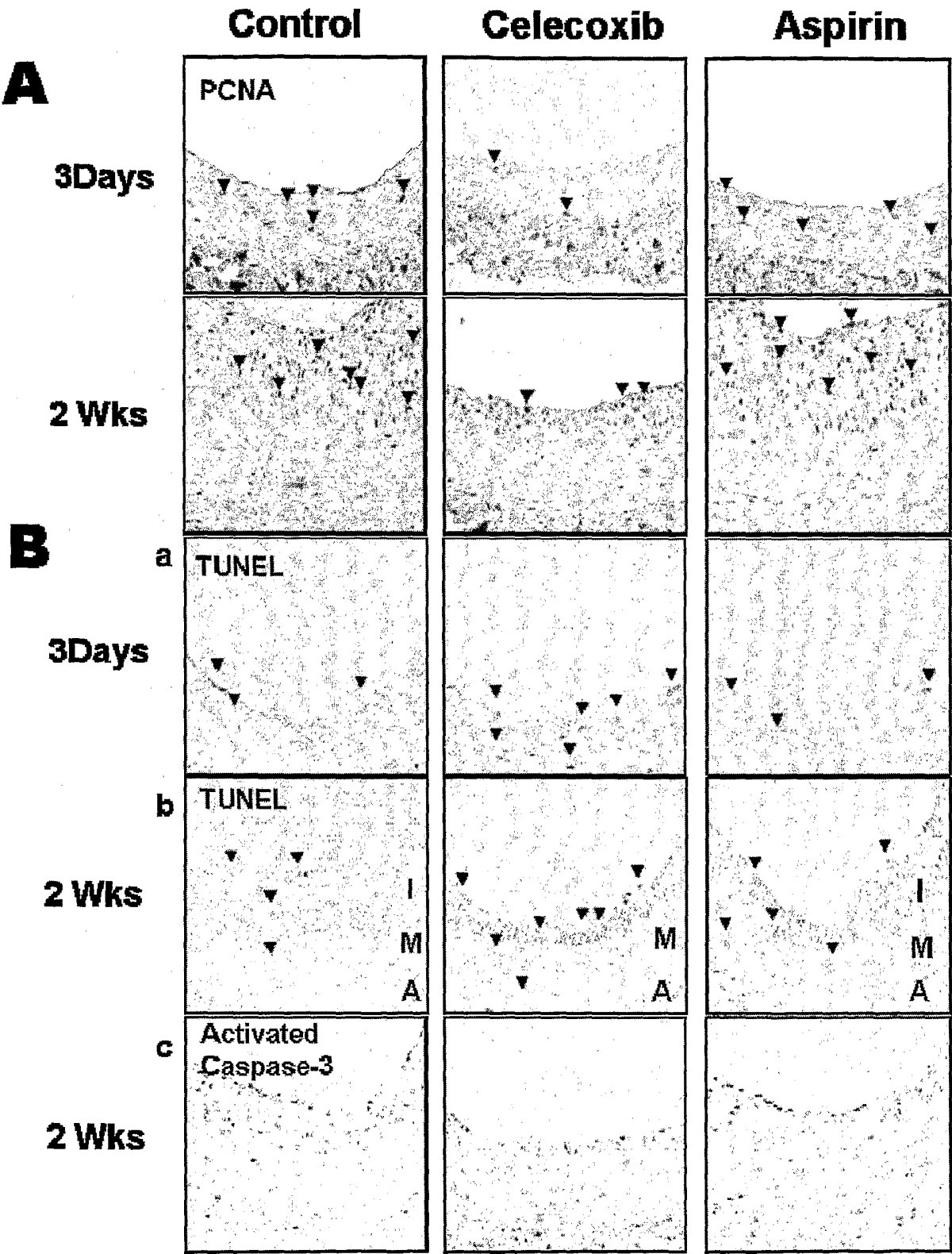
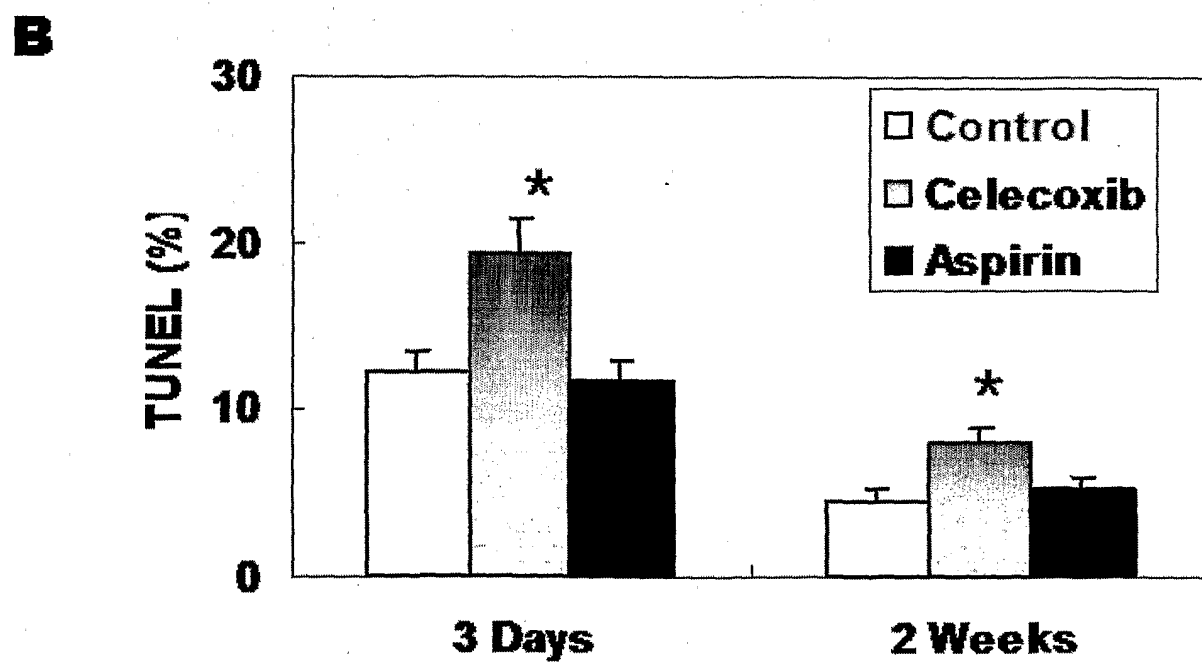
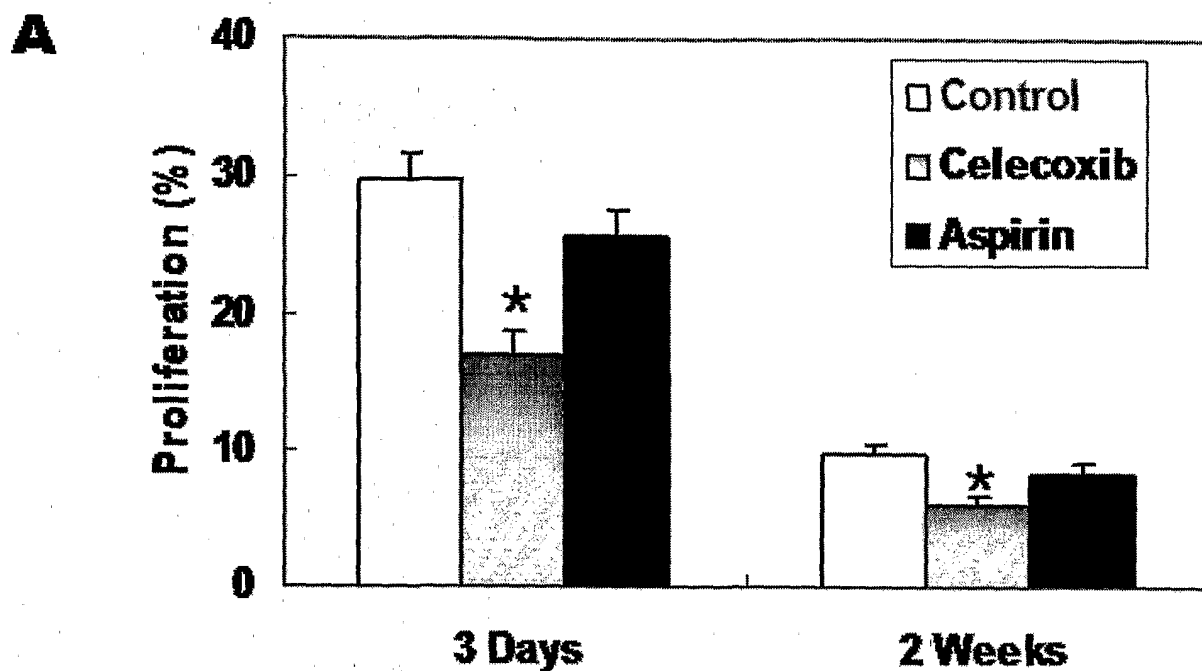
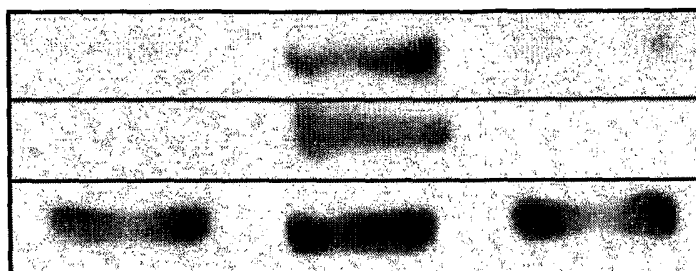
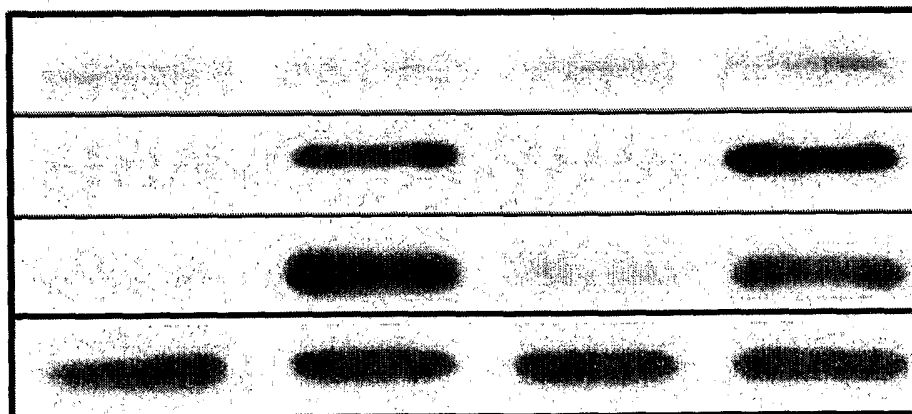
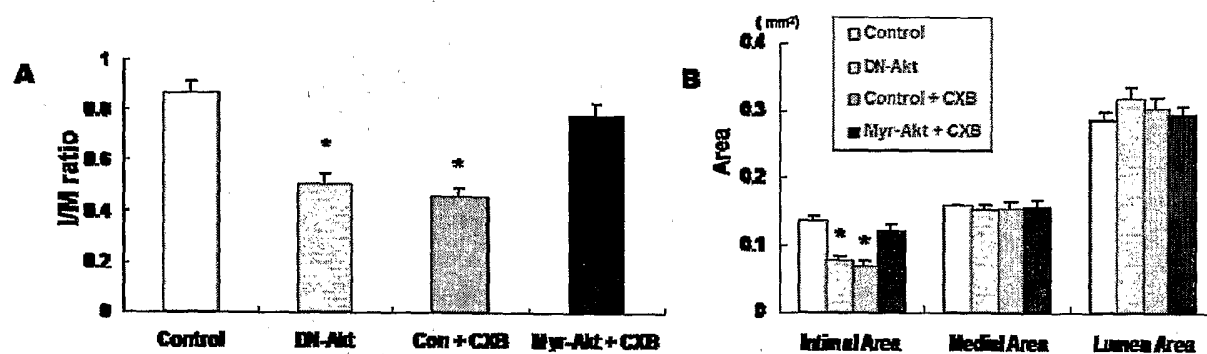
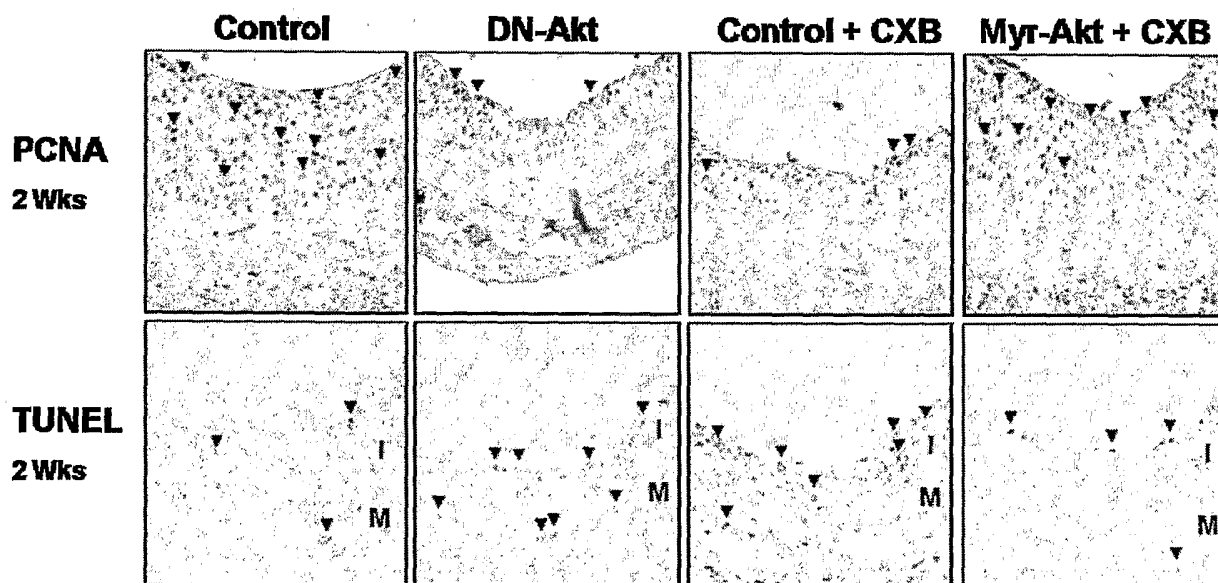
**FIG.5****FIG.6**

FIG.7



**FIG.8**

**FIG.9****A****p-Akt****p-GSK-3 $\beta$**  **$\alpha$ -tubulin****No injury****3 Days****2 Weeks****B****t-Akt****p-Akt****p-GSK-3 $\beta$**  **$\alpha$ -tubulin****No injury****Control****Celecoxib****Aspirin**

**FIG.10****FIG.11**

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/KR2004/002320

**A. CLASSIFICATION OF SUBJECT MATTER****IPC7 A61K 31/415, A61P 9/00**

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)

IPC7 as above

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
Korean patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
Pubmed [(celecoxib OR celebrex) AND (artery restenosis OR neointimal hyperplasia OR Akt inhibition)]

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                            | Relevant to claim No. |
|-----------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | YANG et al., 'Celecoxib, a cyclooxygenase-2 inhibitor, reduces neointimal hyperplasia through inhibition of Akt signaling', Circulation, Jul. 2004, Vol.110(3), pp.301-308.<br>See abstract.                                  | 1 - 3                 |
| X         | US 2003/0109442 A1 (ESPERION THERAPEUTICS, INC.; CEDARS-SINAI MEDICAL CENTER) 12 Jun. 2003<br>See abstract and page 7, left column [0066].                                                                                    | 1 - 3                 |
| A         | WU et al., 'The cyclooxygenase-2 inhibitor celecoxib blocks phosphorylation of Akt and induces apoptosis in human cholangiocarcinoma cells', Mol. Cancer Ther., Mar. 2004, Vol.3(3), pp.299-307<br>See the whole document.    | 1 - 3                 |
| A         | LENG et al., 'Cyclooxygenase-2 promotes hepatocellular carcinoma cell growth through Akt activation: evidence for Akt inhibition in celecoxib-induced apoptosis', Sep. 2003, Vol.38(3), pp.756-768<br>See the whole document. | 1 - 3                 |



Further documents are listed in the continuation of Box C.



See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

08 APRIL 2005 (08.04.2005)

Date of mailing of the international search report

**11 APRIL 2005 (11.04.2005)**

Name and mailing address of the ISA/KR



Korean Intellectual Property Office  
920 Dunsan-dong, Seo-gu, Daejeon 302-701,  
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

LEE, Mi Jeong

Telephone No. 82-42-481-5601



**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

PCT/KR2004/002320

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|-------------------------------------------|---------------------|----------------------------|---------------------|
| US 2003/0109442 A1                        | 12.06.2003          | WO 03026492 A2             | 03.04.2003          |
|                                           |                     | JP 2005504085 T2           | 10.02.2005          |
|                                           |                     | IL 0161110 A0              | 31.08.2004          |
|                                           |                     | EP 1438060 A2              | 21.07.2004          |
|                                           |                     | CA 2460787 AA              | 03.04.2003          |