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Effect of Nimesulide on the Differentiation and Survival of Endothelial Progenitor Cells

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Abstract – Nonsteroidal anti-inflammatory drugs (NSAIDs), particularly the highly selective cyclooxygenase (COX)-2 inhibitors have been shown to decrease the growth of tumor, in part, by inhibition of neovascularization. Recently, besides mature endothelial cells, endothelial progenitor cells (EPCs) have been shown to contribute neovascularization in angiogenic tissues. In this study, we addressed a question whether nimesulide, a selective COX-2 inhibitor, could affect differentiation of EPCs into adhesive endothelial cells *in vitro*. Total mononuclear cells were isolated from cord blood by Ficoll density gradient centrifugation, and then the cells were incubated with nimesulide or vehicle control for 7 days. The number of adherent and spindle-shaped cells decreased by nimesulide treatment in a concentration-dependent fashion at a concentration range of 5 - 200 μM. Moreover, the adherent cells double positive for DiI-ac-LDL uptake and lectin binding significantly decreased upon nimesulide treatment. There was no change of expression of CD31 between treatment and control groups, whereas slight reduction was detected upon treatment in expression of VE-cadherin, ICAM-1, vWF, αv, and α5. Nimesulide also reduced cell viability during first 3 days' culture and induced apoptosis in adherent EPCs, resulting in increased annexin-V-positive and propidium iodide-negative cells. Taken together, these results suggest that nimesulide could be applied for the inhibition of new vessel formation, in part, by inhibiting differentiation and survival of EPCs.

Keywords □ nimesulide, endothelial progenitor cells, nonsteroidal anti-inflammatory drugs (NSAIDs), differentiation, apoptosis

Neovascularization is a key process in the growth of solid tumors and involves the recruitment of endothelial cells to the tumor vascular bed. Two possible sources of endothelialization are 1) endothelial migration and sprouting from preexisting mature endothelial cells, a process called angiogenesis, and 2) recruitment and differentiation of endothelial progenitor cells from circulation. The first process has been well established (Bussolino *et al.*, 1997; Risau, 1997). Vascularization, *in situ* differentiation of endothelial stem cells, was believed to exclusively occur during embryogenesis. However, this concept has been overturned, since Asahara *et al.* (1997) showed that purified CD34-positive cells from adults using MACS system can differentiate *ex vivo* to an endothelial lineage cells, expressing various endothelial markers. Shi *et al.* (1998) also reported the existence of circulating bone marrow-derived endothelial progenitor cells (EPCs) in the adult. Now, the accumulating evidence for the existence of EPC in adult blood has been reported (Shintani *et al.*, 2001; Asahara *et al.*, 1999; Murohara *et al.*, 2000; Boyer *et al.*, 2000). In a previous report, we also confirmed the existence of EPCs in human cord blood (Joe *et al.*, 2002).

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as aspirin and other cyclooxygenase (COX) inhibitors, protect against cancer development and progression (Williams *et al.*,

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1999). Epidemiological studies showed that chronic intake of NSAIDs results in a reduced risk of several malignancies, including colorectal cancer development (Giardiello *et al.*, 1993), and effect may be due, at least in part, to the inhibition of tumor angiogenesis. NSAIDs are known to be potent inhibitors of the two COX isoforms (COX-1 and COX-2), a family of enzymes that catalyze the conversion of arachidonic acid to prostaglandins. COX-2 is inducible by cytokines, growth factors, and tumor promoters, whereas COX-1 is constitutively expressed in most tissue. Particularly, COX-2 is more critically associated with tumor angiogenesis. COX-2 overexpression promotes production of angiogenic growth factors (Tsujii *et al.*, 1998). Williams *et al.* (2000) showed that tumor growth was markedly reduced in COX-2-/-mice compared with COX-1-/- or wild-type mice due to the lack of vessel formation.

Nimesulide (N-(4-nitrol-2-phenoxyphenyl) methanesulfonamide), a selective inhibitor of COX-2 belonging to the sulfonanilide class, has been demonstrated on the anti-inflammatory, analgesic and antipyretic activities in a number of experimental models and in numerous clinical trials (Famaey, 1997). Recently, nimesulide has also been reported to be effective in the suppression of colon carcinogenesis (Fukutake *et al.*, 1998) and the growth of hepatoma or lung tumors (Li *et al.*, 2003; Shaik *et al.*, 2004). However, anti-angiogenic effect of nimusulide has not been studied, although it is used clinically as a selective COX-2 inhibitor. Therefore, we are interested whether nimesulide inhibits EPC differentiation into adhesive vessel cells, thereby inhibits neovascularization required for tumor growth. These results could support the idea that nimesulide could be applied for chemoprevention and combined treatment of cancer as an adjunct without severe toxicity. Here, we examined the effect of nimesulide on EPC differentiation by employing established *in vitro* culture system of EPCs.

MATERIALS AND METHODS

Isolation of mononuclear cells from cord blood

Human umbilical cord blood samples were collected in disposable sterile pyrogefree blood bags (Green cross corp., Yongin, Korea) and used within 4 h. Mononuclear cells (MNCs) were isolated from the blood using the Ficoll-Hypaque density gradient centrifugation method. The blood was diluted with RPMI 1640 (Gibco BRL, Grand Island, NY, USA) and centrifuged at 3,000 rpm for 10 min. The cell layer was diluted with Dulbecco's phosphate-buffered saline (DPBS) with 20 mM EDTA. The sample was added to Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) and centrifuged at 400×g for 35 min. The buffy layer was resuspended in PBS with 20 mM EDTA and centrifuged again at 300×g for 10 min. The cell pellet was washed with Medium 199 (M199, Gibco BRL) and resuspended in the same medium. Clear cells were stained with trypan blue and counted by using a haemacytometer.

Cell culture and treatment of nimesulide

The medium used for the cell culture experiments was M199 supplemented with 10% fetal bovine serum (FBS, Gibco BRL) and heparin (90 μg/ml, Sigma, St. Louis, MO, USA). Mononuclear cells (MNCs) pre-incubated with nimesuldie (Sigma) for 30 min were plated on culture dishes coated with 0.1 mg/ml human fibronectin (Sigma), at a density of 2,500 cells/mm^2. Cells were cultured in a humidified 5% CO_2 incubator at 37°C. After 3 days of culture, non-adherent cells were removed by washing with PBS, and new media and nimesulide were added to each well. The culture was maintained through 7 days and examined for cell number and shape. Adherent cells were counted under phase-contrast microscopy at day 7. Four randomly selected fields per well were evaluated.

Cellular uptake of DiI-labeled ac-LDL and binding of lectin

In order to confirm two EPC phenotypes at once, binding of FITC-labeled *Ulex europaeus* agglutinin (UEA)-1, which is specific for human endothelial cell, and uptake of DiI-ac-LDL, a function associated with endothelial cells, were determined after 7 days of culture. Non-adherent cells were removed by washing with PBS. Adherent cells were incubated with 15 μg/ml DiI-labeled acetylated low density lipoprotein (DiI-ac-LDL, Molecular Probes, Eugene, Oregon) for 1 h at 37°C and later fixed with 1% paraformaldehyde for 10 min. After washes, cells were reacted with 10 μg/ml FITC-labeled UEA-1 (lectin; Sigma) for 1 h. After staining, cells were visualized with an inverted fluorescent microscope. Cells demonstrating double-positive fluorescence were identified as differentiating EPCs.

Immunofluorescence analysis

For analysis of expression of several endothelial markers, the EPCs cultured for 7 days were fixed with 4% paraformaldehyde in PBS for 5 min at R.T.. After permeabilization with 0.1% Triton X-100 (Sigma) in PBS at R.T., cells were washed with washing buffer (0.05% tween 20 in PBS). Nonspecific antibody binding was blocked by incubation in 2% bovine serum albumin (Sigma). Cells were incubated with primary anti-

bodies against KDR/Flk-1 (Santa Cruz Biotechnology, Santa Cruz, CA), CD31 (Dako), vWF (Dako, Glostrup, Denmark) and VE-cadherin (Chemicon, Temecula, CA, USA). After two washes, Cy3 secondary monoclonal antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was applied. Labeled cells were examined under fluorescence scanning microscope (Olympus, Japan).

Fluorescent-activated cell sorting (FACS) analysis

FACS analysis was performed on adherent EPCs cultured for 7 days. After detached with PBS containing 1 mM EDTA, cells were gently scraped off using cell scrapers and washed with 1× PBS. Cells (2×10^5) were incubated in FACS buffer (2% FBS, 1% sodium azide, PBS) for 30 min at 4°C in the presence of monoclonal antibodies against VE-cadherin, vWF, α5, αv, β1, CD54 (ICAM-1) and CD31. Staining of vWF (Dako, Glostrup, Denmark), VE-cadherin (Chemicon, Temecula, CA, USA), α5 (CD49e, BD Bioscience, San Diego, CA, USA), αv (Chemicon) and β1 (Chemicon) was visualized with FITC-conjugated rabbit anti-mouse immunoglobulins (Chemicon). FITC- or PE-conjugated primary antibody was used for the direct detection of CD54 and CD31 (Becton Dickinson, San Jose, CA, USA). After washing, the cells were fixed in 1% paraformaldehyde. Flow cytometric analyses were performed on a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and the data were analyzed using the CELL QUEST analysis program. Histogram of cell number vs. logarithmic fluorescence intensity was recorded for 10,000 cells per sample.

MTS assay

Cell viability assay was performed using the 3-(4, 5-dimethylthiazol-2yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay kit (Promega, Madison, WI), according to the manufacturer's instruction. MNCs isolated from cord blood were plated onto fibronectin-coated 96-well culture plates at a density of 2.5×10^3 cells/mm^2 and incubated in a humidified 5% CO_2 incubator at 37°C. After incubation for 72 h, MTS/PMS solution was added to each well and incubated at 37°C in CO_2 incubator for 4 h. The absorbance was measured at 490 nm using an ELISA reader.

Annexin staining (apoptosis assay)

Cells were grown in fibronectin-coated 6 well plate in M199 supplemented with 10% fetal bovine serum. Cells were then treated with 100 or 250 μM nimesulide for 72 h. After washing in PBS, adherent cells were detached with 1 mM EDTA in PBS. Cells (2×10^5) were stained with Annexin-V-FITC and propidium iodide in 1×binding buffer for 15 min at R.T. using the Annexin-V-FITC kit (BD Bioscience). Flow cytometric analyses were performed on a FACSCalibur flow cytometer and the data were analyzed using the CELL QUEST analysis program.

Data and statistical analysis

All experiments were performed in triplet, sigma plot *t*-test was used to determine statistical significance. A value of $P< 0.05$ was regarded as being statistically significant.

RESULTS AND DISCUSSION

Nimesulide decreased adherent EPC number *in vitro*.

In a previous report, we confirmed that total MNCs isolated from human cord blood gave rise to spindle-shaped and endothelial-like cells when cultured on a fibronectin matrix for 7 days (Joe *et al.*, 2002). EPCs were characterized as adherent cells double positive for DiI-ac-LDL uptake and lectin binding. We also demonstrated by immunocytochemistry that cultured EPCs express several important endothelial lineage markers, including CD31, VE-cadherin, vWF, and KDR/Flk-1. Other molecular features of cultured EPCs were also recently confirmed by fluorescence-activated cell sorter (FACS) analysis (data not shown). Using this established culture system, we examined if nimesulide affects differentiation of endothelial progenitor cells, which exist in a fraction of MNCs of human cord blood.

Incubation of isolated human MNCs with nimesulide for 7 days decreased the number of differentiated, adherent EPCs in a concentration-dependent fashion at a concentration range of 5 - 200 μM (Fig. 1A). The concentration to allow adherent cells to grow at a proposition of 50% compared with control group was about 75 - 100 μM. Most of the adherent cells of the untreated group are spindle-shaped, whereas the adherent cells treated with nimesulide appeared changed cell morphology from spindle shape to shrunken one at more than 50 μM (Fig. 1B).

Evaluation of the effect of nimesulide on EPC differentiation by assessing ac-LDL uptake and lectin binding

To more examine whether nimesulide inhibits differentiation of EPCs, we tested the ability of UEA-1 binding, which is specific for human endothelial cell, and uptake of DiI-ac-LDL, a function associated with endothelial cells. When the cells were

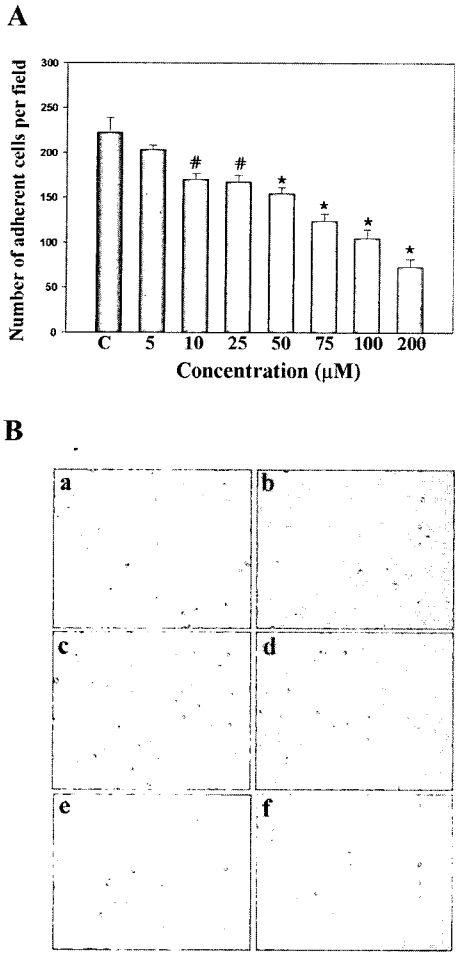

Fig. 1. Effect of nimesulide on the differentiation of EPC into adherent cells. (A) Mononuclear cells were cultured on a fibronectin-coated plate under treatment with nimesulide at indicated concentrations. After 7 days, the number of adherent, differentiated EPCs was counted. The solvent DMSO had no effect on EPC differentiation. (B) The digital microscope images of adherent EPCs treated with nimesulide at various concentrations for 7 days (x 200). a, 0 μM; b, 10 μM; c, 50 μM; d, 75 μM; e, 100 μM; f, 200 μM. The results are mean±SEM from three independent experiments. C; control, # P<0.01, *P<0.001 vs. control.

treated with nimesulide (5-100 μM) for 7 days, the number of adherent cells that took up ac-LDL(+) (red) decreased in a concentration-dependent fashion (Fig. 2A, C), and adherent cells double-positive for ac-LDL(+)- lectin(+) (yellow) also decreased (Fig. 2B, C). At a concentration of 100 μM, the number of adherent cells that took up ac-LDL decreased to the level of 32.3% of that of the cells without treatment. Similarly, adherent cells double positive for ac-LDL uptake and lectin binding decreased to the level of 31.7%. At all the concentrations examined, more than 70% adherent cells were double-positive cells, without any significant variations. IC_{50} was about 75 μM, similar in both assays. Starting from a concentration of 10 μM, the number of adherent, double-positive cells began to significantly reduce. Therefore, significant reduction, although not maximal inhibition, can be reached within clinically achievable concentrations (up to 30 μM), which seem to be lower than the maximum tolerated dose (Warrington *et al.*, 1993). Collecting together, we could conclude that nimesulide treatment can reduce the differentiation of EPCs to endothelial-like cells.

Analysis of expression of endothelial lineage markers

To examine whether treatment with nimesulide affect expression of endothelial lineage phenotypes, we analyzed the expression level of endothelial lineage markers on adherent cells after treatment by immunofluorescence microscopy. Expression of CD31, VE-cadherin, vWF, and KDR/Flk-1 was examined. As shown in Fig. 3A, all the enodothelial markers examined were expressed in most of the adherent cells after incubation for 7 days in a control group. When treated with nimesulide at several concentrations (25 μM, 50 μM and 100 μM), the adherent cells were modestly stained with CD31, VE-cadherin, KDR/Flk-1, or vWF antibody, without a significant difference of staining density between treatment and control groups. The number of cells stained with CD31, VE-cadherin, vWF, and KDR/Flk-1 was decreased dose-dependently, in proportion to the number of total adherent cells. Inhibition of nimesulide on gene expression of endothelial markers could not be determined quantitatively in this method. Cells were stained with the antibody against CD31 (>95%), vWF (>95%), or VE-cadherin (>85%) without any significant differences between treatment and control groups.

Statins enhance cell surface expression of α5 and β1 integrin subunits as well as αvβ5 integrin, and thereby improve neovascularization in ischemic tissue (Walter *et al.*, 2002). In case of NSAIDs, they also have been implicated in integrin activity, for an example, by inhibiting αvβ3-dependent activation of the small GTPases Cdc42 and Rac, resulting in inhibiting of endothelial-cell spreading and migration *in vitro* (Dormand *et al.*, 2001). Indomethacin and aspirin also inhibit platelet aggregation by suppressing activation of integrin αIIβ3 (Dominguez-Jimenez *et al.*, 1999). Piroxicam and meloxicam suppress cytokine-induced activation of leukocyte β1 and β2 integrins (Garcia-Vicuna *et al.*, 1997). Therefore, we examined whether nimesulide treatment at a concentration of 50 μM affects expression of adhesion surface marker molecules such as VE-cadherin, CD54 (ICAM-1), CD31 (PECAM-1), and vascular

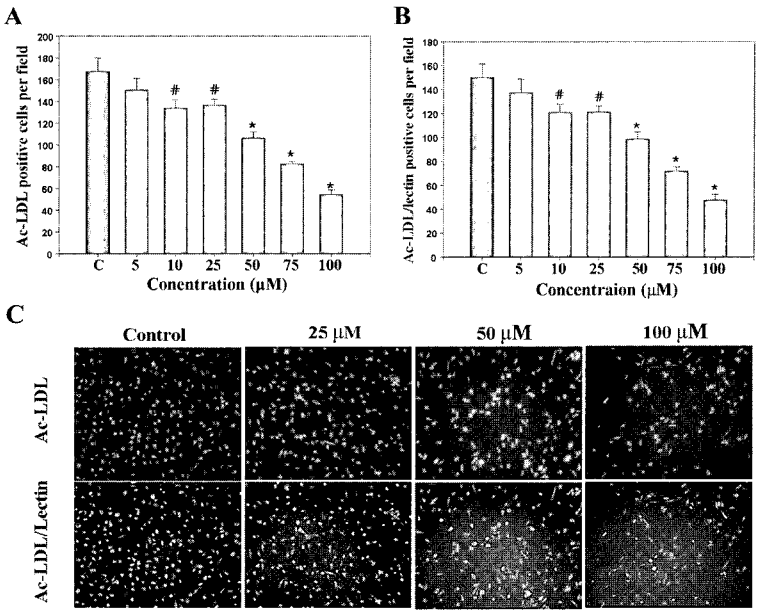

Fig. 2. Nimesulide decreases the number of adherent EPCs double positive for ac-LDL uptake and lectin binding. After MNCs were incubated on fibronectin-coated plates with nimesulide at various concentrations for 7 days, ac-LDL positive adherent cells (A) and double positive cells (B) were counted, respectively. The solvent DMSO had no effect on EPC differentiation. (C) Digital microscopic images of adherent cells stained for ac-LDL uptake (red) and lectin binding (green) were presented (×100). Double positive cells appear yellow in the overlay. C; control, # $P<0.05$, *$P<0.001$ vs. control.

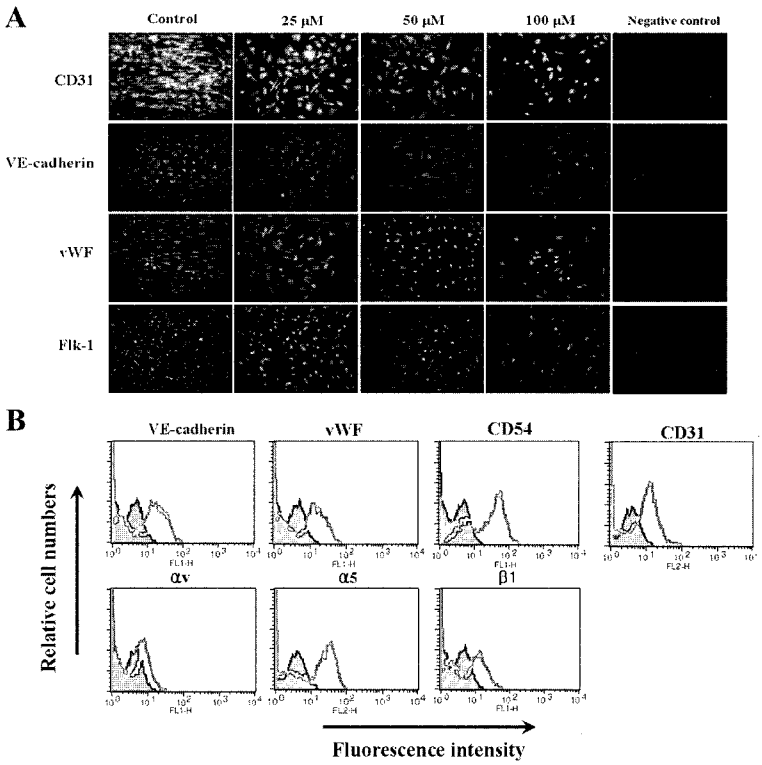

Fig. 3. Immunofluorescence and FACS assay of nimesulide-treated EPCs. (A) Adherent cells cultured for 7 days under treatment of nimesulide at various concentrations were subjected to immunofluorescence to analyze the expression of KDR/Flk-1, CD31, vWF and VE-cadherin. Secondary antibody (Cy3) was used as a negative control. (B) Changes on surface receptor expression of EPCs grown in the absence or presence of nimesulide (50 μM) for 7 days (FACS analysis) were shown by representative histograms. Dark area, mouse IgG control-labeled cells; gray line, untreated cells (no nimesulide); and black line, cells exposed to nimesulide of 50 μM. Histrograms represent cell number (y-axis) versus fluorescent intensity (x-axis, log scale).

integrins (αv, α5, and β1). Expression of CD31 was hardly changed, and expression of adhesion molecules such as VE-cadherin, CD54 (ICAM-1), integrin subunit αv, and α5 was slightly reduced by nimesulide treatment (Fig. 3B). From these results, we concluded that nimesulide could in part affect expression of adhesion molecules, without resulting in any dramatic effect on EPC differentiation.

Effect of nimesulide on cell viability

To assess the effect of nimesulide on cell viability, a MTS assay was performed for total mononuclear cells cultured for first 3 days. Each well contains adherent and non-adherent cells. In this assay, nimesulide inhibited cell viability of adherent EPCs and floating non-EPCs in a dosedependent manner with IC_{50} of about 100 μM (Fig. 4A). We also checked whether nimesulide induces apoptosis in cultured EPCs, since it has been reported that nimesulide induces apoptosis independently from COX-2 *in vitro* and *in vivo* (Eibl *et al.*, 2003; Hida *et al.*, 2000). FACS analysis was performed to detect annexinV positive and PI-negative cells representing apoptosis for the adherent cells after first 3 days culture with treatment of 100 or 250 mM nimesulide. As shown in Fig. 4B, nimesulide induced apoptosis in adherent EPCs. However, no significant difference in necrotic cells was detected between 100 μM and 250 μM (7.15 % and 6.72%, respectively). These results suggest that the induction of apoptosis by nimesulide might play an important role in leading to reduction of adherent, differentiated EPCs, although other possible mechanisms could not be excluded.

From these results, we can conclude that nimesulide can inhibit EPC differentiation, in part by inducing apoptosis. Other possibilities including inhibition of COX-2 and thereby inhibition of VEGF or PGE_2, or activation of peroxisome proliferation-activated receptor-γ could not be excluded for the explanation of inhibitory effect of nimesulide on EPC differentiation (Shaik *et al.*, 2004), since the IC_{50} obtained for COX-2 inhibition are varied in a range of 0.22~90.3 μM in *in vitro* models (Famaey, 1997). More detailed studies are likely necessary for figuring out the precise mechanism underling inhibitory effects of nimesulide in the future.

In conclusion, we suggest that nimesulide could be used for the inhibition of new vessel formation by inducing apoptosis and inhibiting differentiation of endothelial progenitor cells, thereby being applied for chemoprevention of cancer or combined therapy for cancer patient with conventional chemotherapeutic agent.

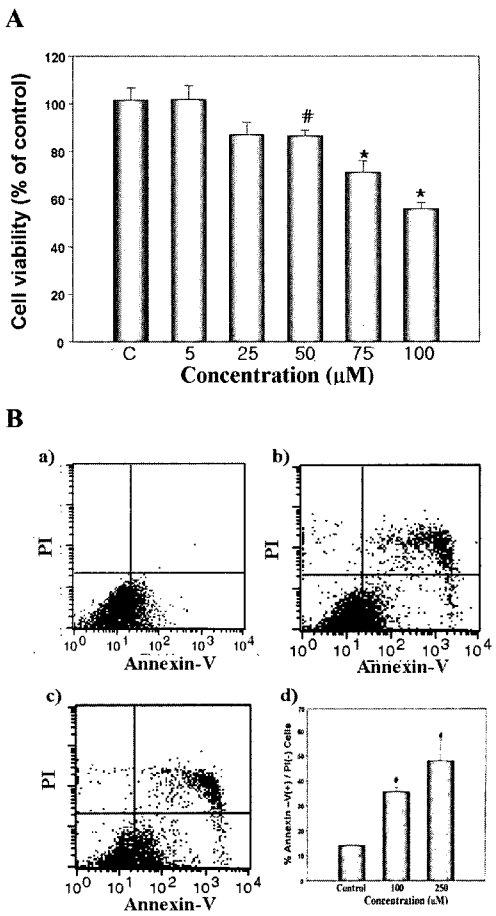

Fig. 4. (A) Cell viability assay. MNCs were treated with nimesulide at the indicated concentrations. After 72 h, both adherent and non-adherent cells were subjected to MTS assay. (B) The effect of nimesulide on induction of apoptosis in the adherent EPCs was determined by flow cytometry. Cells were incubated with 0 μM (a), 100 μM (b), or 250 μM (c) nimesulide for 72 h, and adherent cells were subjected to FACS analysis after staining with annexin-V-FITC and PI. Annexin-V-positive, propidium iodide-negative cells, representing the apoptotic subpopulation, cluster in the right lower quadrant. Vertical and horizontal axes show the staining intensity for propidium iodide and annexin-V, respectively. Number of apoptotic cells derived form FACS data was compared between treated and control groups in a graph (d). C; control, #P<0.05, *P<0.01 vs. control.

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