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ENDOTHELIAL CELL PROLIFERATION MAY BE MEDIATED VIA THE PRODUCTION OF ENDOGENOUS LIPOXYGENASE METABOLITES

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SUMMARY: Endogenous regulators of endothelial cell proliferation have not been clearly defined. We investigated whether the cyclooxygenase and/or lipoxygenase metabolites are involved in this process, and report that lipoxygenase products can modulate endothelial cell growth. Nordihydroguaiaretic acid - a lipoxygenase inhibitor, inhibited endothelial cell proliferation as well as DNA synthesis. 5,8,11,14-Eicosatetraynoic acid - an inhibitor of both lipoxygenase and cyclooxygenase also inhibited endothelial cell DNA synthesis, while indomethacin - a selective cyclooxygenase inhibitor did not affect cell proliferation or DNA synthesis. While arachidonic acid stimulated DNA synthesis, this effect was completely abolished by nordihydroguaiaretic acid. These results demonstrate that products of the lipoxygenase pathway can affect endothelial cell proliferation.

The formation and growth of new capillaries is termed angiogenesis or neovascularization. Sequential steps in this process include degradation of capillary basement membrane, the migration and proliferation of endothelial cells and their final alignment into capillary sprouts(1). Factors that stimulate these events have been identified in the growth medium of various tumor cells and tissue extracts(2). Previous reports have suggested that the metabolites of arachidonic acid [both prostaglandins and hydroxyeicosatetraenoic acids (HETEs)] can modulate the process of angiogenesis by affecting endothelial cell migration in vitro and promoting the formation of capillaries in vivo(3-5). Our present study was conducted to investigate whether cyclooxygenase and/or lipoxygenase metabolites were involved in mediating endothelial cell proliferation. We provide evidence that endothelial cell proliferation can be mediated via products of the lipoxygenase pathway.

Abbreviations: MEM-10, minimal essential medium supplemented with 10% fetal calf serum; NDGA, nordihydroguaiaretic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; HETE, hydroxy-eicosatetraenoic acid.

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MATERIALS AND METHODS

Culture of Endothelial cells: Fetal bovine aortic endothelial cells were kindly provided by Dr. Bert Glaser and Dr. Janet Graeber (Johns Hopkins Medical Center, Baltimore, MD). The cells were cultured in minimal essential medium supplemented with 10% fetal calf serum (MEM-10), and grown in 75 cm^2 flasks at 37°C in a humidified atmosphere of 5% CO_2 in air(6). Cells from passages 11 to 18 were used in the experiments to be described, each passage representing two cell doublings.

Cell proliferation assays: Endothelial cells from stock cultures were plated at a density of 2.5 to 4 x 10^4 cells per 9 cm^2 well in six well plates containing MEM-10. Cell numbers varied by <5% among the wells of an individual plate. Following a 22 hour incubation, the medium was replaced with fresh MEM-10 containing the indicated concentration of indomethacin or nordihydroguaiaretic acid (NDGA). Cultures were incubated for the indicated times, and the cells were then released by trypsin (0.05%) - EDTA (0.02%). Cell number was determined using a Coulter Counter Model ZB1 (Coulter Electronics, Inc., Hialeah, FL).

[^{3}H]Thymidine Incorporation into DNA: Endothelial cells (4 x 10^4 cells/well) were seeded into 6 well plates. After 20 to 23 hours of incubation, the medium was replaced with either control MEM-10 or MEM-10 containing the indicated concentrations of the test compounds. The cells were preincubated in that medium for 15 min, [^{3}H]thymidine (5 uCi) was then added, and cells incubated for an additional 60 min. Media were then removed, cells washed twice with ice cold phosphate buffered saline and detached by trypsinization. Trichloroacetic acid was added to a final concentration of 5% and following incubation at 0°C for 30 min, the trichloroacetic acid precipitated material was collected on 0.2 micron Millipore filters (Millipore Corp., Bedford, MA) which were dried and counted in ACS scintillant (Amersham Corp., Arlington Heights, IL).

[^{3}H]Uridine and [^{14}C]Leucine Incorporation: Incorporation of[^{3}H]uridine (2u Ci/ml of medium) into RNA, and [^{14}C]leucine (0.8 uCi/ml of medium) into protein was determined as described above for [^{3}H]thymidine incorporation into DNA.

Statistical Analysis: Statistical evaluation was performed by the paired Student's t-test when only one treatment group was involved. The significance of difference in a treatment series was determined by randomized complete block analysis of variance (F-ratio)(7). Individual treatments in a treatment series were compared with the control by the Dunnett's test.

Materials: Arachidonic acid (purity >99%) was obtained from Nu-Chek,. Inc. (Elysian, MS). NDGA and indomethacin were purchased from Sigma Chemical Co. (St. Louis, MO). 5,8,11,14-Eicosatetraynoic acid (ETYA) was a generous gift from Dr. J.E. Pike (Upjohn Co.). [6-^{3}H]Thymidine (28.9 Ci/mmol), [5-^{3}H]uridine (29 Ci/mmol), and L [U-^{14}C]leucine (348 mCi/mmol) were obtained from Amersham (Arlington Heights, IL). Tissue culture supplies were obtained from Gibco (Grand Island, NY).

RESULTS

Effects of NDGA and Indomethacin on Cell Proliferation:

Fig. 1 compares the effects of indomethacin, a cyclooxygenase inhibitor,(8) and NDGA a lipoxygenase inhibitor(9), on the

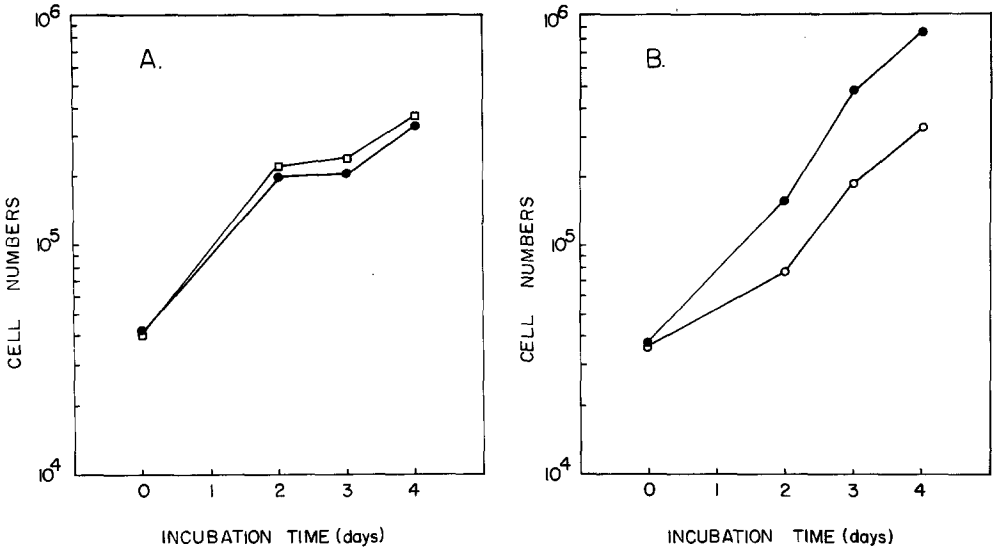

Fig. 1. Effects of Indomethacin (A) and NDGA (B) on Endothelial Cell Proliferation: Following overnight incubation, cells were treated with fresh control MEM-10 (●) or in that medium containing 100 uM indomethacin (□), or 15 uM NDGA (○). Cultures were incubated for the times indicated, cells were then released by trypsinization and counted. Values represent the means of duplicate determinations from a representative experiment. Deviations from mean values were <5%. Similar results were observed in three different experiments performed in duplicate.

proliferation of fetal bovine aortic endothelial cells in culture. Indomethacin (100 uM) had no effect, while NDGA (15 uM) decreased cell proliferation. In six further experiments we have shown that the inhibition of cell proliferation by NDGA was dose-dependent (Fig. 2). After 5 days of incubation with 15 uM and 20 uM NDGA, cell growth was significantly inhibited by 40 ± 9% (1SE; $P < 0.05$) and 47 ± 11% ($P < 0.01$) respectively. Although inhibition of 11 ± 2% and 25 ± 5% was observed at 5 and 10 uM NDGA respectively, the results were not statistically significant at the latter concentration.

Effects of NDGA, ETYA and Indomethacin on [^{3}H]Thymidine [^{3}H]Uridine and [^{14}C]Leucine Incorporation: As shown in figure 3, panel A, NDGA (20 uM) significantly decreased the incorporation of [^{3}H]thymidine into DNA (50%, $P < 0.01$). No significant inhibition of [^{3}H]uridine incorporation into RNA or [^{14}C]leucine incorporation into protein was observed (Fig. 4). The effects of ETYA [an inhibitor of cyclooxygenase and lipoxygenase(10)] and indomethacin (a cyclooxygenase inhibitor) on [^{3}H]Thymidine incorporation were also determined. ETYA significantly inhibited thymidine incorporation (47%; $P < 0.01$

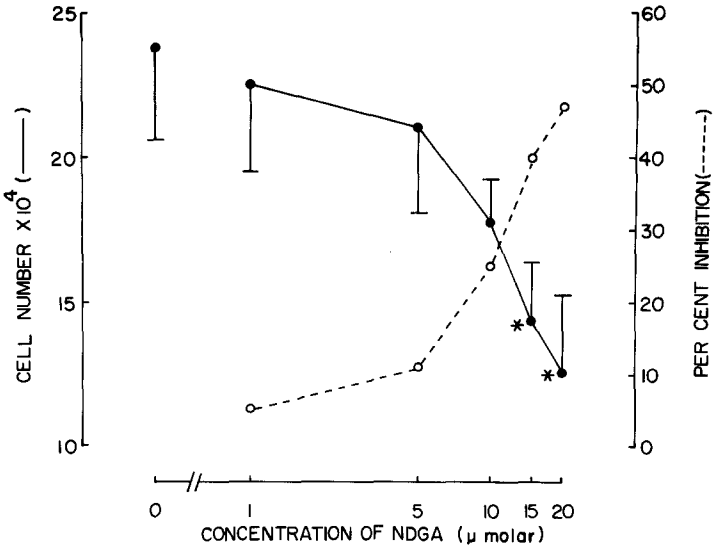

Fig. 2. Concentration-dependent Inhibition by NDGA of Endothelial Cell Growth: Following overnight incubation, endothelial cells were treated with fresh MEM-10 containing the indicated concentrations of NDGA (1-20 uM). Cells were counted after a 5 day incubation period. Values represent the mean ± 1SE of six experiments. *The cell numbers at 15 and 20 uM NDGA were significantly different from control at $P < 0.05$ and $P < 0.01$ respectively.

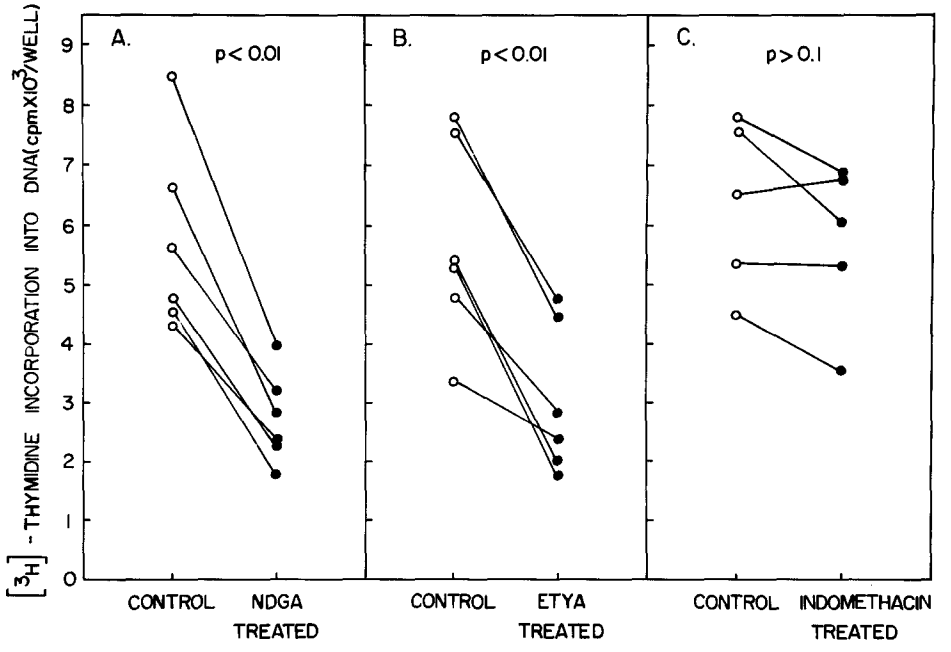

Fig. 3. Effect of NDGA, ETYA and Indomethacin on [^{3}H]Thymidine Incorporation into DNA: Endothelial cells were preincubated for 15 min in 1 ml of fresh MEM-10 (control) or in that medium containing 20 uM NDGA (panel A), 33 uM ETYA (panel B) or 100 uM indomethacin (panel C). [^{3}H]Thymidine (5 uCi per well) was added and the incubation was continued for a further 60 min. DNA was isolated and radioactivity determined as described in "Materials and Methods".

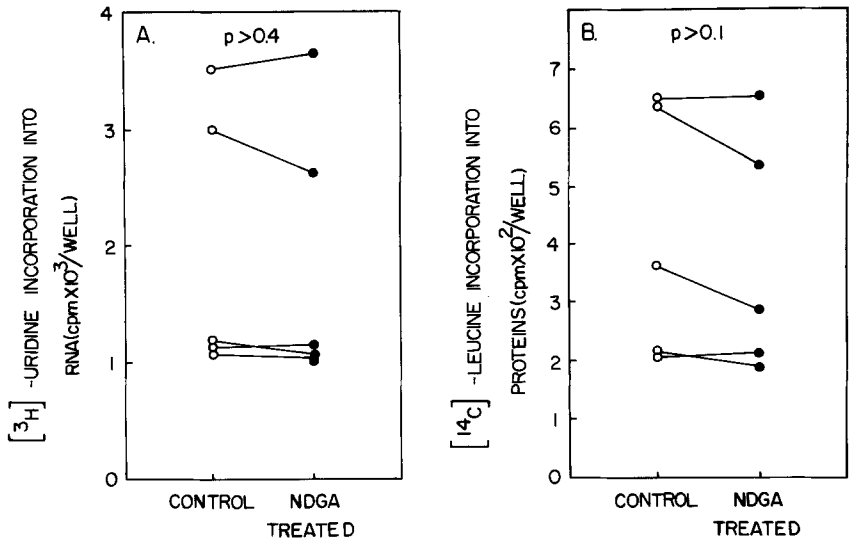

Fig. 4. Effect of NDGA on [^{3}H]Uridine and [^{14}C]Leucine incorporation into RNA and Protein: Endothelial cells were preincubated for 15 min in 1 ml of fresh MEM-10 (control) or in that medium containing 20 uM NDGA. 2 uCi [^{3}H]uridine (panel A) and 0.8 uCi [^{14}C]leucine (panel B) were added, and the incubation was continued for a further 60 min. RNA and protein were isolated and radioactivity determined as described in "Materials and Methods".

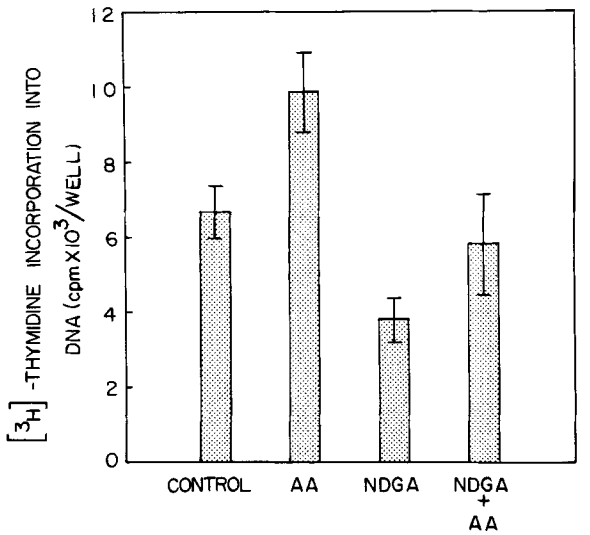

Fig. 5. Effect of Arachidonic acid, NDGA, or Arachidonic acid plus NDGA on [^{3}H]Thymidine Incorporation into DNA: Endothelial cells were preincubated for 15 min with 1 ml fresh MEM-10 (control), or in that medium containing arachidonic acid (AA)(90 uM), 20 uM NDGA, or 90 uM arachidonic acid plus 20 uM NDGA. [^{3}H]Thymidine (5 uCi) was then added. Following a 60 min incubation period, DNA was isolated and radioactivity determined as described in "Materials and Methods". Values are the mean $\pm$ 1SE of six determinations. Stimulation of DNA synthesis by arachidonic acid was significantly enhanced when compared to control ($P < 0.05$). While NDGA inhibited basal DNA synthesis ($P<0.05$), NDGA also abolished the enhanced DNA synthesis observed in the presence of arachidonic acid.

Fig. 3, panel B) whereas indomethacin had no effect (Fig. 3, panel C). ETYA and indomethacin did not significantly affect either RNA or protein synthesis (data not shown).

Inhibition by NDGA of Arachidonic Acid Stimulated [^{3}H]Thymidine Incorporation: Arachidonic acid (90 uM) stimulated DNA synthesis by 55 $\pm$ 23% when compared to control ($P < 0.05$; Fig. 5). While NDGA inhibited basal DNA synthesis in endothelial cells (43 $\pm$ 6%; $P < 0.05$) the arachidonate stimulated DNA synthesis was completely abolished by NDGA (89 $\pm$ 25% control; P=NS).

DISCUSSION

In this study we evaluated the effects of indomethacin, NDGA, and ETYA-inhibitors of arachidonic acid metabolism, on endothelial cell proliferation and DNA synthesis, in order to ascertain whether arachidonic acid metabolites were involved in mediating cell growth. The results presented indicate that endogenous lipoxygenase products modulate endothelial cell proliferation. This conclusion is supported by the inhibition of endothelial cell growth by NDGA-a lipoxygenase inhibitor, the inhibition of DNA synthesis by NDGA, and ETYA - an inhibitor of both cyclooxygenase and lipoxygenases, and the inhibition by NDGA of arachidonic acid stimulated synthesis of DNA. Since indomethacin had no effect on either cell proliferation or DNA synthesis, we also conclude that cyclooxygenase metabolites are not involved in the regulation of endothelial cell growth.

Endothelial cells isolated from human umbilical veins and bovine aortas have been shown to convert arachidonic acid via the lipoxygenase pathway to several mono-hydroxy derivatives including 15-HETE, 12-HETE and 5-HETE, with 15-HETE being the major mono-HETE produced by these cells(11-13). In addition to 15-HETE, bovine aortic endothelial cells synthesize several di-HETEs (our unpublished data). The identity of these presumed di-HETEs is not complete. It is possible that one or more of these lipoxygenase metabolites of arachidonic acid may be involved in mediating the proliferative responses in endothelial cells.

Lipoxygenase products are involved in the mediation of cellular responses to several physiologic stimuli. Lipoxygenase inhibitors block the bile salt stimulated synthesis of epithelial cell DNA(14) and the endotoxin stimulated activation of mouse peritoneal macrophages(15). NDGA blocks prolactin

induced casein and lipid biosynthesis in cultured mouse mammary gland explants(16), and both NDGA and ETYA have been shown to inhibit stimulated histamine release by human leucocytes(17). A preliminary report has suggested that the proliferation of K562 human leukemia cells is decreased by inhibitors of lipoxygenase activity(18). Our results demonstrate this effect in normal endothelial cells. Thus the lipoxygenase pathway appears to be essential to a variety of cellular regulatory mechanisms both in normal and neoplastic cells.

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