Effects of Nicardipine on Tube Formation of Bovine Vascular Endothelial Cells In Vitro

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Background and Purpose: The purpose of this study was to assess the effect of nicardipine, a Ca\(^{2+}\) channel blocker, on angiogenesis in vitro.

Methods: Bovine carotid artery endothelial cells were cultured between type I collagen gel layers with 10\(^{-6}\) to 10\(^{-3}\) M nicardipine. The morphological changes were monitored by phase-contrast microscopy and photographed. The total length of tubular structures was measured with an image analyzer system. Endothelial proliferation and migration assays were also performed with the same doses of nicardipine.

Results: Cultured endothelial cells form tubular structures between collagen gel layers. Tube formation of endothelial cells was suppressed by culture with 10\(^{-3}\) to 10\(^{-2}\) M nicardipine in a dose-dependent manner. Migration of endothelial cells was also suppressed by the same doses of nicardipine. However, proliferation of endothelial cells was not enhanced.

Conclusions: Nicardipine acts as an inhibitor of angiogenesis in vitro by inhibiting the migration of endothelial cells. This result suggests that nicardipine may have therapeutic potential in angiogenic disorders such as tumor growth, atherogenesis, and diabetic retinopathy. (Stroke 1992;23:1637–1642)

KEY WORDS • angiogenesis • endothelium • nicardipine • cattle

Angiogenesis is crucial in pathophysiological events, such as progressive tumor growth, the formation of collaterals to ischemic tissues, and the process of wound healing. However, because this phenomenon is a multifactorial event including endothelial cell proliferation, migration, and morphological change in the formation of vascular networks, it has been difficult to investigate each step of angiogenesis. Recent advances in cell culture techniques provide a useful method to investigate each process of angiogenesis. Angiogenesis in vitro may be expressed as a tube or tubular structure of endothelial cells, the total length of which may be objectively evaluated. Although many angiogenic factors have been investigated in vitro,\(^{1–11}\) the mechanism of angiogenesis is still unclear.

Recent investigations have revealed that Ca\(^{2+}\) metabolism of vascular endothelial cells and smooth muscle cells plays an important role in modulating not only vascular tone but atherogenesis.\(^{12–14}\) This suggests that Ca\(^{2+}\) metabolism may have profound effects on various endothelial functions. We investigated the effect of nicardipine on angiogenesis using cultured endothelial cells.

Materials and Methods

Bovine carotid artery endothelial cells were isolated as described previously.\(^{15}\) Briefly, the bovine carotid artery was obtained from the local slaughterhouse. Extension tubes (Japan Medical Supply, Hiroshima, Japan) were inserted into both ends of the artery and tied with wire. All branches of the artery were clamped. The artery was perfused with Dulbecco's phosphate-buffered saline (PBS) (Nissui, Tokyo, Japan) to wash out the blood. Then one of the tubes was clamped, PBS containing 0.25% trypsin (Difco Laboratories, Detroit, Mich.) and 0.02% ethylenediaminetetraacetic acid (EDTA) (Nacalai Tesque, Kyoto, Japan) was infused from the other side, and the tube was clamped. The artery was placed in a beaker containing PBS and incubated at 37°C for 15 minutes. Then the trypsin solution was flushed from the artery with PBS, collected in a 50-ml conical centrifuge tube (Falcon Plastics, Oxnard, Calif.), inactivated with fetal calf serum (FCS) (Bioproducts, Inc., Whittaker, Mass.), and centrifuged at 1,000 rpm for 5 minutes. The pellet was suspended in Dulbecco's modified Eagle's medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% FCS, penicillin G (200 units/ml), and streptomycin (200 \(\mu\)g/ml). Then cells were seeded onto 35-mm petri dishes (Falcon Plastics) that contained small fragments of cover glasses and incubated in 5% CO\(_2\) and 95% air. This procedure was repeated several times.

The medium was exchanged every 3 days. When colonies of endothelial cells were recognized, fragments of cover glasses that had only one colony were picked up and transferred to 12-well culture dishes. Endothelial cells were identified by incorporation of low density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (Dil-Ac-LDL) (Biomedical Technologies, Inc., Stoughton, Mass.).
The experiment of angiogenesis in vitro was performed according to the method of Kanayasu et al.\textsuperscript{10} Vitrogen 100 (Collagen Co., Calif.), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer containing 0.1N NaOH, and 10-fold concentrated minimal essential medium (Nissui, Tokyo, Japan) were mixed at a ratio of 8:1:1 and incubated in 12-well culture dishes at 37°C for 1 hour to make the gels. Bovine carotid artery endothelial cells were inoculated at a density of 1.5 × 10\textsuperscript{5} cells per well with 1 ml of medium containing 10% FCS and incubated in 5% CO\textsubscript{2} and 95% air for 24 hours. Then the medium was aspirated, 1 ml of collagen gel was overlaid, and 1 ml of medium supplemented with 10% FCS and several concentrations of nicardipine hydrochloride (Yamanouchi Pharmaceutical Co., Tokyo, Japan) were applied to the medium, which was then incubated. The medium was exchanged every 2–3 days. The morphological changes were monitored by phase-contrast microscopy and photographed five times at random. The tubular structure was traced and transcribed from the photomicrograph to the tracing paper for quantification. Total length of tubular structure was measured by an image analyzer. The assay was performed in triplicate. Student’s \textit{t} test was used for statistical analysis.

For transmission electron microscopy, samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 1 hour at 4°C and postfixed in 1% OsO\textsubscript{4} for 1 hour. The samples were dehydrated with graded ethanol and embedded in Epon 812. Ultrathin sections were cut with an ultramicrotome, stained with uranyl acetate and lead citrate, and examined under a JEOL 1200EX electron microscope.

To investigate the effect of nicardipine on the proliferation of bovine carotid artery endothelial cells, cells were inoculated at a density of 5.0 × 10\textsuperscript{4} cells per well in 24 culture dishes and incubated with medium containing 0.5% FCS, 200 units/ml penicillin G, 200 \mu g/ml streptomycin, and various concentrations of nicardipine at 37°C in 5% CO\textsubscript{2} and 95% air. The medium was changed every 2 days. Cells were trypsinized after 7 days and counted with a hemocytometer. The assay was performed in triplicate.

The migration of bovine carotid artery endothelial cells was assayed as reported previously.\textsuperscript{10,16} Briefly, the lower wells of the blind well chambers (Neuroprobe, Bethesda, Md.) were filled with serial dilutions of nicardipine in medium containing 10% FCS and covered with Nucleopore chemotaxis membranes of 8.0-\mu m pore size (Nucleopore Co.). They were inoculated at a density of 1.0 × 10\textsuperscript{4} cells per well in the upper wells and incubated at 37°C in 5% CO\textsubscript{2} and 95% air for 3 hours. Then the membrane was removed, and cells on the upper surface of the membrane were wiped off. Cells were fixed with 90% ethanol, stained with hematoxylin, and mounted on a slide glass, and total cells on the lower surface of the membrane were counted. The assay was performed in triplicate.

Results

Bovine carotid artery endothelial cells presented a typical cobblestone appearance and incorporated Dil-Ac-LDL (Figure 1).

Figure 2 depicts bovine carotid artery endothelial cells cultured between collagen gel layers after 5 days of culture. When the cells were overlaid with a second layer of collagen gel, they rapidly began to elongate and formed tubular structures within 2 days; these structures developed gradually (Figure 2, top left). These micrographs show the morphological change that occurred with 10\textsuperscript{-7} to 10\textsuperscript{-5} M nicardipine. Addition of 10\textsuperscript{-7} M nicardipine to the medium caused development of tubular structures, but many cells did not form these structures (Figure 2, bottom left). With an increase in
FIGURE 2. Phase-contrast micrographs of bovine carotid artery endothelial cells cultured between collagen gel layers at 5 days of culture. Top left panel: Control; bottom left panel: $1.0 \times 10^{-7} M$ nicardipine; top right panel: $1.0 \times 10^{-6} M$ nicardipine; bottom right panel: $1.0 \times 10^{-5} M$ nicardipine. Magnification, ×97.5. Bar=200 μm.
the concentration of nicardipine, the formation of tubular structures was suppressed (Figure 2, top right and bottom right). Figure 3 is a transmission electron micrograph of the tubular structures, showing the lumen surrounded by endothelial cells. The total length of tubular structures after 10 days of culture was measured (Figure 4). Tubular length of the control group was 1.07±0.09 mm/mm². Cells cultured with 10⁻⁷ M nicardipine formed tubular structures the total length of which was 1.09±0.16 mm/mm². However, as is shown in Figure 4, the total length of tubular structures decreased with the increase in the concentration of nicardipine (0.90±0.11, 0.65±0.09, 0.39±0.10, and 0.36±0.07 mm/mm² at 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M, respectively).

The proliferation of bovine carotid artery endothelial cells was not affected by 10⁻⁵ to 10⁻⁷ M nicardipine.

The effect of nicardipine on the migration of bovine carotid artery endothelial cells was assayed by counting the cells that traversed the membrane from the upper surface to the lower surface, which was in contact with the chamber containing nicardipine. Nicardipine significantly inhibited the migration at 10⁻⁷ M in a dose-dependent manner, as demonstrated in Figure 5.

**Discussion**

Angiogenesis is a multifactorial phenomenon involving endothelial cell proliferation, migration, and morphological change. Recently each step of angiogenesis was investigated using an in vitro model of angiogenesis. Angiogenesis of endothelial cells in an in vitro model may be expressed in terms of tube or capillary formation and can be statistically analyzed by measuring the total length of the tube with an image analyzer system. Previous studies have revealed that extracellular matrices have profound effects on tube formation of endothelial cells in the culture system. Present findings also suggest that bovine carotid artery endothelial cells form tubular structures when a second layer of type I collagen gel is overlaid. Many soluble angiogenic factors have also been investigated with a culture system. Other factors, such as phorbol esters, leukotriene C₄, or...
The present findings clearly demonstrated that nicardipine, a Ca$^{2+}$ channel blocker, exhibited an angi-inhibiting effect on cultured endothelial cells by inhibiting the migration of endothelial cells without affecting the proliferation of endothelial cells. This indicates that endothelial cells have voltage-operated Ca$^{2+}$ channels and is compatible with the recent investigation that dihydropyridines prevent the release of endothelium-derived relaxing factor. Additionally, Ca$^{2+}$ metabolism has relevance to endothelial migration, which is modulated by Ca$^{2+}$ channel blockers. The role of Ca$^{2+}$ metabolism in angiogenesis should be further investigated.

Other effects of the Ca$^{2+}$ channel blockers on endothelial cells or smooth muscle cells have been studied in a culture system. Hirosumi et al demonstrated that nifedipine suppressed the increase of intracellular Ca$^{2+}$ of porcine aorta endothelial cells provoked by superoxide anions, which are thought to be risk factors of arteriosclerosis. Nakao et al demonstrated that nicardipine suppressed the migration of cultured vascular smooth muscle cells. The effect of Ca$^{2+}$ channel blockers on inhibition of vascular smooth muscle cell proliferation has also been studied. All these effects act to inhibit the development of atherosclerosis. Interestingly, angiogenesis occurs in the atheromatous plaques and may play an important role in the development of atherosclerosis. Therefore, there is a possibility that Ca$^{2+}$ channel blockers may have antiatherosclerotic action by modulating angiogenesis in atheromatous plaques.

Because these findings were obtained by an in vitro model using nicardipine for a very short time, it is uncertain whether these results are true in vivo and whether other types of Ca$^{2+}$ channel blockers also have the same effects on cultured endothelial cells. However, the present findings suggest that tube formation of endothelial cells has some relevance to Ca$^{2+}$ metabolism and that Ca$^{2+}$ channel blockers play important roles in regulating neovascularization. Ca$^{2+}$ channel blockers may have therapeutic potential in angiogenic disorders such as progressive tumor growth, arteriogenesis, diabetic retinopathy, and rheumatoid arthritis.

### References

This communication by Kaneko et al suggests the potential novel use of the drug nicardipine as an inhibitor of angiogenesis. Through the use of a simple yet elegant endothelial culture model resulting in endothelial tube formation complemented by various in vitro assays, the authors have provided compelling evidence that nicardipine can act as an inhibitor of endothelial migration while having no effect upon proliferation. As most now concur that endothelial migration is an initiating step in the process of angiogenesis, this finding would suggest that nicardipine may have some therapeutic role in the modulation of the angiogenic processes that occur with tumor growth, wound repair, and inflammation. Obviously, as noted by the authors, in vivo confirmation of this antimigratory effect is required to complement these in vitro findings. However, if indeed these findings hold in vivo, the potential utility of nicardipine and/or other calcium channel blockers in blunting angiogenesis should be the focus of continued laboratory investigation.

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*Stroke*. 1992;23:1637-1642
doi: 10.1161/01.STR.23.11.1637

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/23/11/1637

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