Cell Growth and Na-K-Cl Cotransport Responses of Vascular Smooth Muscle Cells of Milan Rats

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Abstract The present study examines the role of serum growth factors in the proliferative response and Na-K-Cl cotransport activity of vascular smooth muscle cells from Milan normotensive (MNS) and hypertensive (MHS) rats. Cells from thoracic aorta of both strains were cultured in 10% serum medium and made quiescent by 72 hours in 0.3% serum medium. MHS cells grown with 10% serum had a shorter population doubling time than MNS cells between passages 8 and 12 (13.8±1.7 versus 20.1±1.6 hours, P<.01, n=4). MHS cells also exhibited a higher response of thymidine incorporation into nucleic acid to serum, epidermal, and platelet-derived growth factor BB. In MHS cells epidermal (100 ng/mL) and platelet (50 ng/mL) growth factors increased thymidine incorporation 2- and 10-fold, respectively. In MNS cells epidermal factor did not induce a significant response, and that of platelet factor was twofold lower than in MHS cells. Binding curves revealed a higher number of receptors for platelet than epidermal growth factor in both strains and a similar number of both receptors in MHS and MNS cells. Quantitative immunoblotting of these receptors...
Cellular Growth Curves

VSMCs were maintained during 48 hours in DMEM and 10% FBS (GIBCO) and then were induced to a quiescent state by replacing the serum with 0.3% serum. After 72 hours the cells were stimulated to grow with 10% FBS medium and removed by trypsin-EDTA every 24 hours for counting in a Neubauer chamber.

Thymidine Incorporation Into DNA

VSMCs plated at 60,000 cells per 35-mm well were allowed to reach 80% confluence in DMEM/10% FBS, kept quiescent for 48 hours in DMEM/0.1% bovine serum albumin (BSA), and incubated during 24 hours with FBS (0.3% or 10%), EGF (100 ng/mL), or PDGF (100 ng/mL) (both from Austral Biologicals). They were then pulse-labeled for 2 hours with [3H]thymidine (1 μCi/mL, 10–4 mol/L thymidine) (Amersham) and washed three times with 1 mL ice-cold phosphate-buffered solution (PBS), twice with 1 mL 10% trichloroacetic acid for 10 minutes, and twice with ice-cold methanol. The radioactivity associated with DNA was determined after extraction with 1 mL 0.05% sodium dodecyl sulfate (SDS) and 0.05% NaOH; cells were processed for β-counting and protein determinations as described.

Na-K-Cl Cotransport and Na-K Pump Activities

Cotransport was measured with the use of [32P] as a tracer for K+ influx inhibitable by bumetanide. Na-K pump activity was measured as ouabain-sensitive Rb+ influx. VSMCs plated on 35-mm dishes were equilibrated for 120 minutes in balanced salt solution at 37°C. Rb+ influx was initiated by replacing the balanced salt solution with an Rb+-rich (5 μCi/mL) (Amersham) solution containing (mmol/L) NaCl 140, KCl 5, CaCl₂ 2, with and without ouabain 2, and bumetanide 0.1. The radioactive solution was removed at different times, the dishes were rapidly washed three times with ice-cold PBS, and maintained quiescent for 48 hours, then medium was removed and replaced by 0.3% or 10% DMEM. Cell number or total protein was measured every 24 hours. Hypertensive rat VSMCs cultured in medium including 10% serum grew to cell densities significantly greater (P<.01) than normotensive rat cells. Cells were studied at passage 6 for normotensive and 5 for hypertensive cells.

EGF and PDGF Binding Sites

[125I]-EGF was prepared by the chloramine-T method as described. PDGF BB was prepared by the method of Bolton and Hunter with some modifications. Cells were seeded at 20,000 cells per well in 12 multiwell plastic dishes (Elkay Products, Inc), grown to confluence in DMEM/10% FBS, and maintained quiescent for 48 hours in DMEM/0.1% BSA before the binding. After two washings with 1 mL PBS the cells were incubated at 4°C for 2 hours to reach equil-

Immunoblots of EGF and PDGF BB

Receptor Proteins

The EGF receptor protein was analyzed by immunodetection in blots of proteins separated by SDS-polyacrylamide gel electrophoresis as described. The HK2 antibodies, which cross-react with the rat EGF receptor, were kindly provided by Dr J. Schlessinger (New York Medical Center). Antibodies to the extracellular domain of the PDGF BB receptor produced by recombinant technology were from Austral Biologicals. Extracts of cells grown to 80% confluence in 10-cm dishes were kept for 48 hours in DMEM/0.1% BSA and prepared exactly as described, including the antiproteases cocktail. After the extracts were adjusted to the same amount of protein, the glycoprotein receptors were precipitated with 200 μL pre-washed concanavalin A-Sepharose beads (Pharmacia LKB Biotechnology); this step increased the sensitivity of the detection and reduced the background. The pellet was suspended in loading buffer and electrophoresed on an SDS-polyacrylamide gel. The immunocomplexes were developed with protein A–alkaline phosphatase as described in detail in our previous report.

Results

Growth Kinetics of VSMCs of Milan Rats

VSMCs were maintained in low-serum media for 72 hours without apparent loss in cell number. Fig 1 shows representative experiments carried out in MNS and MHS cells grown in 10% and 0.3% serum at passage 5. At a low serum concentration, VSMCs of both strains grew slowly, whereas MHS cells proliferated at a faster rate than MNS cells in 10% serum. In four paired experiments performed on four different preparations at less than passage 8, the population doubling time was 24.5±2.1 hours for MHS and 34.2±2.1 hours for MNS cells (P<.001). The faster proliferation rate of MHS cells was also observed between passages 8 and 14, but the population doubling time was shorter. Doubling times, calculated on the slope of the growth curve between 24 and 72 hours after addition of 10% serum, were 20.1±1.6 hours for MNS and 13.8±1.7 hours for MHS cells (n=5 for each strain, P<.01). Cells from both
MHS cells also responded differently to serum in thymidine incorporation into DNA (data not shown). MHS cells increased thymidine incorporation per milligram protein 12-fold when switched from 0.3% to 10% serum, whereas MNS cells increased thymidine incorporation threefold to fourfold. These findings led us to explore the effect of growth factors present in serum that could be responsible for the higher proliferative responsiveness of MHS cells. Enhanced thymidine incorporation was observed in quiescent MHS cells incubated with EGF and PDGF BB (Fig 2). PDGF BB stimulation of DNA synthesis after a period of quiescence was greater in MHS cells than MNS cells by 12-fold and fourfold to sixfold, respectively (Fig 2). In another four experiments the response of thymidine incorporation was determined per milligram of cell protein. PDGF BB (50 ng/mL) and 10% serum increased thymidine incorporation 12-fold in MHS cells and fivefold in MNS cells. The dose response of thymidine incorporation per milligram protein had a half-maximal response at 20 ng/mL for MHS and 10 ng/mL for MNS cells (data not shown). EGF doubled the level of thymidine incorporation, with no consistent difference between rat strains. There was no additional effect when both factors were added together at 100 ng/mL (Fig 2).

**EGF and PDGF BB Binding Sites**

To estimate the receptor density on the cell surface of VSMCs, we determined saturation binding kinetics for EGF and PDGF BB receptors using 125I-labeled ligands at 4°C. Fig 3A shows one of four experiments on EGF binding in cells of both strains. Scatchard analysis of EGF binding (plot not shown) was used to determine $B_{max}$ and $K_d$ in each experiment, and the mean±SEM was calculated for the four experiments. As shown in Fig 3A, MHS cells expressed a lower binding activity per cell than MNS cells. The mean $B_{max}$ values for the four experiments were 4340±145 binding sites per cell for MHS cells compared with 7400±163 binding sites per cell for MNS cells (n=4). The binding affinity was similar for both cell types (0.73±0.15 and 1.08±0.04 nmol/L, MNS versus MHS cells). However, MHS cells are smaller than MNS cells and have 4 µg protein per 10,000 cells, whereas MNS cells are larger and have 10 µg protein per 10,000 cells. When the density of receptors was normalized per milligram of cell protein, these differences were no longer significant. PDGF BB binding kinetics showed (Fig 3B) that VSMCs of both strains had 5.8-fold more PDGF BB receptors than EGF receptors. In two experiments, the number of PDGF BB receptors calculated by a Scatchard plot was lower in MHS cells (20,000±3000 per cell) than MNS cells (40,000±5100 per cell) but of similar density when expressed per milligram of cell protein.

Measurements of EGF receptor protein by immunoblots gave similar results as ligand binding studies. As shown in Fig 4, a higher amount of EGF receptors was detected in MNS cells when the immunoblot analysis was made on electrophoretically separated protein corresponding to 1.5×10⁶ cells. When immunoblots were made by loading the same amount of protein of MNS and MHS cells, there was no apparent difference in the number of receptors per milligram of cell protein between these strains. Similar results were obtained in immunoblots of the PDGF receptor protein (Fig 4); a higher amount of PDGF receptors per cell was found in MNS cells (Fig 4), but no difference was found between
MHS

- + - + - +

MNS

- + - + - +

anti-EGF-R

anti:PDGF-R

Fig 4. Immunoblots show epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) BB receptor (R) proteins present in vascular smooth muscle cells from hypertensive (MHS) and normotensive (MNS) Milan rats. Amount of EGF and PDGF receptor protein from MHS (lanes 2, 4, and 6) and MNS (lanes 1, 3, and 5) cells was assessed by immunoblots comparing the same number of cells (1.5×10⁶ cells) for EGF receptors (lanes 1 and 2) and PDGF BB receptors (lanes 5 and 6). MNS cells contain higher amounts of each receptor on a per cell basis. Similar amounts of receptor proteins are found when the comparison is made loading blots with the same amount of EGF receptor protein (lanes 3 and 4) (1.3 mg). For this experiment the detection of PDGF BB receptors in MHS cells was under the sensitivity of the method (lane 6).

Na-K-Cl Cotransport and Na-K Pump Activity in MNS and MHS Cells

VSMCs grown to confluence with 10% serum showed higher Na-K-Cl cotransport activity in the MHS than MNS strain (P<.001) below passage 8 and between passages 8 and 12 (Fig 5). In the log phase of serum-stimulated cell growth, bumetanide-sensitive Rb⁺ influx also was significantly higher in MHS than MNS cells (Fig 6). In contrast, cotransport activity did not show significant differences in quiescent VSMCs of both strains (Fig 6). The enhanced responsiveness of quiescent MHS cells to PDGF BB led us to study the effect of this mitogen on Na-K-Cl cotransport activity. PDGF BB stimulated bumetanide-sensitive Rb⁺ influx more in MHS than MNS cells (Fig 6). In the log phase, cotransport activity was stimulated by PDGF BB twofold more in MHS cells (13 U, P<.01) than in MNS cells (7.6 U, P<.01). EGF did not have any effect on cotransport (data not shown). Ouabain-sensitive Rb⁺ influx did not show significant differences between strains (data not shown).

Discussion

Cultured VSMCs from MHS rats showed a higher proliferative response to serum than VSMCs from the normotensive strain. In addition, quiescent cells from MHS rats showed a higher responsiveness of thymidine incorporation to serum, PDGF BB, and EGF than MNS cells. PDGF BB is a competence growth factor, a powerful mitogen for VSMCs, and a Ca²⁺ mobilizing agonist. Both strains had 5.7-fold more PDGF BB than EGF receptors. Although the mechanism for the different proliferative response of MHS cells remains unclear, it is not due to differences in the density of binding sites per cell for these receptors. Both EGF and PDGF BB showed a higher number of binding sites per cell in the MNS than MHS cells; these differences are caused by the smaller volume of MHS cells. These findings in Milan rats differ from those reported for cultured VSMCs from SHR and Wistar-Kyoto (WKY) rats. The density of EGF receptors assayed by radioligand binding was twofold higher in cells from SHR than those from WKY rats, but the responsiveness of thymidine incorporation to EGF was similar for these strains, and only in the presence of 0.5% serum did EGF have a larger effect in the growth kinetics of SHR cells. However, MHS cells with a lower density of EGF receptors per cell than MNS cells responded similarly to EGF, and SHR cells, with a higher EGF receptor density than WKY cells, do not have a higher responsiveness to EGF. Therefore, the density of EGF receptors does not correlate with effects on thymidine incorpor-
poration in VSMCs of the hypertensive rats. This is in contrast to transfected NIH 3T3 cell lines expressing a large number of EGFR receptors, which show a stimulation of DNA synthesis that correlates with the number of receptors per cell.\(^{12}\) Hadrava et al\(^{12}\) also reported that the responsiveness to a mixture of PDGF isoforms of thymidine incorporation into DNA was higher in SHR than WKY cells. Cultured VSMCs can return to a synthetic phenotype that can be accompanied by an increase in the production and secretion of PDGF-like mitogens as well as expression of different ratios of PDGF BB and AB receptors.\(^{18}\) Human VSMCs express threefold more PDGF BB than PDGF AB receptors,\(^{19}\) and thymidine incorporation responded well to PDGF BB but poorly to PDGF AA; however, Swiss 3T3 cells, with about an equal number of BB and AB receptors, responded equally well to both isoforms. These findings led us to hypothesize that the high mitogenic responsiveness to PDGF BB of hypertensive cells might be related to expression of distinct proportions of either the AB or BB receptor isoforms. PDGF BB can partially occupy AB receptors, which are not titrated by the radioligand assay or the immunoblots. Alternatively, the different responsiveness to this growth factor may rely in the signal-transducing system beyond the receptor or in the signaling for its degradation. The responses of Ca\(^{2+}\) movement to the three isoforms of PDGF (AA, BB, and AB) appear to be different: the AB isoform is more able to elevate cytosolic Ca\(^{2+}\) mainly by stimulation of Ca\(^{2+}\) influx, and the BB isoform by inducing Ca\(^{2+}\) mobilization.\(^{2,20}\) In fact, MHS cells have higher cytosolic Ca\(^{2+}\) than MNS cells,\(^{7}\) a characteristic possibly related to expression of a different proportion of AB and BB receptors.

Serum-grown MHS cells showed a higher Na-K-Cl cotransport activity than MNS cells at confluence and in the log phase of serum growth, whereas transport activity was similar in the quiescent state. In addition, MHS cells in the quiescent state and log phase of growth responded to PDGF BB with a greater enhancement of cotransport activity than MNS cells. This cotransporter is activated by cell volume reduction to increase cell volume. An intriguing possibility is that the proliferation of cells of small volume might not enhance peripheral resistance as well as proliferation of cells of normal volume. To our knowledge, this is the first report of regulation of cotransport by PDGF BB, which is most likely triggered to compensate for the small cell volume of MHS cells. The signal transduction of the PDGF BB receptors that regulate Na-K-Cl cotransport remains to be determined, but modulation by protein kinase C, Ang II, or cyclic AMP has been shown to vary with the cell type and species.\(^{21}\) Studies in serum-grown VSMCs from SHR and WKY rats showed a lower cotransport activity\(^{22}\) and higher Na\(^{+}\)-H\(^{+}\) exchange activity\(^{10}\) in the hypertensive strain. It therefore seems that ion transport alterations in VSMCs of hypertensive rat models are reflecting the differences in their responsiveness to regulatory mechanisms controlled by PDGF BB and Ang II receptors.

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