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

Mitzy Canessa, Gloria Salazar, Erica Werner, Gino Vallega, Alfonso Gonzalez

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<0.01, 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 immunoblot of these receptors from the MHS strain revealed a twofold lower number of platelet than epidermal growth factor receptors. Cotransport activity (bumetanide-sensitive *Rb influx in nanomoles per milligram protein per 5 minutes) was found to be significantly higher in MHS cells (16±3, n=18) than MNS cells (8±3, n=15) at confluence as well as in the log phase of serum-stimulated growth. No differences were found between strains with cells in the quiescent state. However, platelet growth factor (50 ng/mL) markedly stimulated cotransport in quiescent MHS cells, whereas epidermal growth factor was without effect. In conclusion, MHS cells exhibited enhanced serum- and platelet factor-stimulated proliferation rates and cotransport activity compared with MNS cells. These results suggest that platelet factor BB plays an important role in the proliferation of hypertensive vascular cells. (Hypertension. 1994;23[part 2]:1022-1026.)

Key Words • Milan rats • PDGF BB • smooth muscle • cell proliferation • Na-K-Cl cotransport

Vascular smooth muscle cells (VSMCs) within the tunica media of adult arteries are in quiescent state of growth, with the cell cycle reversibly arrested in the G1/G0 phase. In rats, the replication rate is approximately 0.02%, but this slow growth rate can be altered by mechanical injury and the release of growth factors by platelets, monocytes, and endothelial cells. The initiation of the proliferative cycle and the rate of growth are controlled by autocrine and paracrine secretion of growth factors and the expression of these receptors.

Platelet-derived growth factor (PDGF), isoform BB, is the most powerful mitogenic for the initiation of the proliferative cycle in VSMCs, and it may play a role in the hyperplasia of the tunica media in hypertension. Stimulation of ion fluxes is among the earliest changes induced by growth factor addition to G0-arrested cells. Activation of Na+-H+ exchange by several mitogens has been well characterized in various cell types, and stimulation of Ca2+-Na+ exchange by PDGF BB and of Na-K-Cl cotransport by epidermal growth factor (EGF) has been reported in VSMCs and fibroblasts. Previous investigations have shown that VSMCs from hypertensive rats also exhibited enhanced proliferation, with spontaneously hypertensive rats (SHR) having a higher number of EGF receptors and enhanced responsiveness of Na+-H+ exchange to angiotensin II (Ang II). This abnormality may be responsible for the greater vascular hyperplasia observed in genetic models of rat hypertension and partially account for the high vascular resistance to blood flow. In contrast to SHR, VSMCs of Milan hypertensive (MHS) rats exhibit a blunted response of Na+-H+ exchange to Ang II and enhanced growth, but their responsiveness to growth factors has not yet been studied. The MHS strain is a low-renin hypertensive model with kidney abnormalities and blunted vessel response to vasoconstrictors. The present study was designed to investigate in VSMCs from Milan rats the role of serum and PDGF BB in cellular proliferation and expression of Na-K-Cl cotransport activity.

Methods

Cell Isolation and Culture

VSMCs were isolated by enzymatic dissociation from thoracic aorta of 2-month-old MHS and Milan normotensive (MNS) rats as previously described. The Milan rats were kindly provided by Dr Giuseppe Bianchi and Patrizia Ferrari from Sigma-Tau, Milan. Four separate preparations of four rats of each strain were used in this study. VSMCs were cultured under 5% CO2 in 75-cm2 culture flasks containing Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS) supplemented with penicillin (100 U/mL), and streptomycin (100 μg/mL). VSMCs were made quiescent.
by 72-hour incubation with 0.3% FBS/DMEM medium or were cultured with 10% serum for growth curves, thymidine incorporation, and cotransport measurements.

**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 during 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 \( ^{3}H \)-thymidine (1 μCi/mL, 10\(^{-4} \) mol/L thymidine) (Amersham), and washed three times with 1 mL ice-cold phosphate-buffered saline (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 0.5% sodium dodecyl sulfate (SDS) and 0.05% NaOH; cells were processed for \( \beta \)-counting and protein determinations as described.

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

Cotransport was measured with the use of \(^{86}\)Rb 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. \(^{86}\)Rb influx was initiated by replacing the balanced salt solution with an \(^{86}\)Rb-rich (5 μCi/mL) (Amersham) solution containing (mmol/L) NaCl 140, KCl 5, CaCl\(_2\) 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 0.1% SDS for \( \gamma \)-radioactivity counting and protein content as described.\(^{9}\) The cell-associated counts per minute was divided by the specific activity of the \(^{86}\)Rb-K\(^+\) influx solution to normalize the flux in nanomoles per milligram protein per 5 minutes. The influx was found to be linear up to 15 minutes.

**EGF and PDGF Binding Sites**

\(^{125}\)I-EGF was prepared by the chloramine-T method as described.\(^{11,12}\) \(^{125}\)I-PDGF BB was prepared by the method of Bolton and Hunter\(^{13}\) with some modifications. Cells were seeded at 20,000 cells per well in 12 multwell plastic dishes (Ekalay 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 equilibrium conditions with increasing concentrations of the corresponding labeled ligand in 1 mL Hanks solution, 25 mmol/L HEPES, pH 7.4, and 0.1% BSA. Unbound ligand was removed by washing four times with 1 mL PBS. The cells were solubilized in 0.3 mL 1 N NaOH, and the associated radioactivity was determined in a gamma counter. Nonspecific binding was determined by incubation in the presence of a high excess (1 μg/mL) of unlabeled ligand; nonspecific binding was 20% for EGF and 50% for PDGF BB.

**Immunoblot 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.\(^{14,15}\) RK2 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,\(^{16}\) 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.\(^{17}\)

**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 34.8 ± 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
strains 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 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 \( B_{\text{max}} \) 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 \( B_{\text{max}} \) 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 \( \mu \)g protein per 10 000 cells, whereas MNS cells are larger and have 10 \( \mu \)g 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 (26 000±3000 per cell) than MNS cells (42 000±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 4A, a higher amount of EGF receptors was detected in MNS cells when the immunoblot analysis was made on electrophoretically separated protein corresponding to 1.5×10^6 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 4B); a higher amount of PDGF receptors per cell was found in MNS cells (Fig 4), but no difference was found between
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FIG 5. Bar graphs show Na-K-Cl cotransport activity of serum-grown vascular smooth muscle cells derived from hypertensive (MHS) and normotensive (MNS) Milan rats. Cells from both strains were grown with 10% serum and measured at confluence. Cotransport activity was measured as bumetanide-sensitive Rb+ influx in nanomoles per milligram protein (prot) per 5 minutes as described in “Methods.” Below passage 8, cotransport activity for MNS cells was 8±3 (n=15) and for MHS cells, 16±3 (n=18). Higher than passage 8, cotransport activity for MNS cells was 24.5±2.5 (n=12) and for MHS cells, 32±6 (n=9). MHS cells exhibit higher cotransport activity than MNS cells independent of the passage number. Probability values by unpaired t test.

FIG 6. Bar graphs show Na-K-Cl cotransport activity of vascular smooth muscle cells derived from normotensive (MNS) and hypertensive (MHS) Milan rats. Cells from both strains were made quiescent by culture in 0.3% serum medium for 72 hours and grown with 10% serum to the log phase for cotransport measurements. Effect of platelet-derived growth factor (PDGF) BB (50 ng/mL) was studied in both growth states. Cotransport activity is not significantly different in quiescent cells but is significantly higher in MHS cells during the log phase of growth (P<.01). Quiescent cells of MHS rats treated with PDGF BB showed significantly higher cotransport activity than MNS cells (P<.01, paired t test). BS indicates bumetanide-sensitive.

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 incor-
portation in VSMCs of the hypertensive rats. This is in contrast to transfected NIH 3T3 cell lines expressing a large number of EGF receptors, which show a stimulation of DNA synthesis that correlates with the number of receptors per cell.12 Hadrava et al 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 Ca2+ movement to the three isoforms of PDGF (AA, BB, and AB) appear to be different: the AB isoform is more able to elevate cytosolic Ca2+ mainly by stimulation of Ca2+ influx, and the BB isoform by inducing Ca2+ mobilization.20 In fact, MHS cells have higher cytosolic Ca2+ 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 stage 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 activity22 and higher Na+/H+ exchange activity20 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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