Transforming Growth Factor \( \beta_1 \) Expression and Effect in Aortic Smooth Muscle Cells From Spontaneously Hypertensive Rats

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Previous studies demonstrated that in addition to an increased response to growth factors, cultured vascular smooth muscle cells derived from spontaneously hypertensive rats (SHRs) grow to a greater density than cells from normotensive Wistar-Kyoto (WKY) rats. Transforming growth factor \( \beta_1 \) (TGF-\( \beta_1 \)) has a bimodal effect on vascular smooth muscle cell growth, depending on cell density. The present study investigated the relation between cell density and expression of the proto-oncogene \( c-fos \) and TGF-\( \beta_1 \) in cells from WKY rats and SHRs. The results demonstrate an increased accumulation of \( c-fos \) mRNA in calf serum-stimulated SHR cells but only at a high cell density. The expression of TGF-\( \beta_1 \) mRNA was enhanced in growing SHR cells at every density studied as early as 24 hours after inoculation, with a further increase at later times. The effect of exogenous TGF-\( \beta_1 \) on new DNA synthesis was evaluated by \(^{[3H]} \) thymidine incorporation. At a low cell density, TGF-\( \beta_1 \) had no effect on DNA synthesis in either WKY or SHR vascular smooth muscle cells. At a high cell density, there was a significant increase of DNA synthesis in response to TGF-\( \beta_1 \) in SHR cells without any effect in WKY cells. In conclusion, contact inhibition of vascular smooth muscle cells from SHRs at a higher cell density is accompanied by an earlier expression of the marker gene \( c-fos \) and preceded by an exaggerated expression of TGF-\( \beta_1 \). Considered together with the stimulating effect of exogenous TGF-\( \beta_1 \) at a high cell density, the results suggest an abnormal feedback control (autocrine stimulation) of this growth factor and its involvement in altered contact inhibition of vascular smooth muscle cells from SHRs. (Hypertension 1991;17:896–901)

Abnormal vascular smooth muscle cell (VSMC) growth has been suggested to be one of the significant contributors to increased peripheral vascular resistance in hypertension.\(^1,2\) Several studies, including ours, have demonstrated greater VSMC proliferation in spontaneously hypertensive rats (SHRs) when compared with normotensive Wistar-Kyoto (WKY) controls in response to growth stimuli, such as calf serum, epidermal growth factor, and platelet-derived growth factor (PDGF).\(^3,6\) The expression of this intermediate phenotype appears to be stable up to the 20th passage. An additional intermediate phenotype of this abnormal growth is an increased specific growth rate, which is most apparent when cells approach confluency.\(^6\) It has been suggested that the production of an extracellular matrix may be essential for the expression of enhanced VSMC proliferation in SHRs.\(^7\) Transforming growth factor \( \beta_1 \) (TGF-\( \beta_1 \)) has been shown to stimulate the expression and synthesis of several extracellular matrix proteins.\(^8,9\) It also possesses multiple and complex regulatory functions that influence cell growth.\(^10\) TGF-\( \beta_1 \) is present in significant amounts in platelets\(^11,12\) and is synthesized by a variety of cells in culture.\(^13\) This factor acts as a bifunctional modulator of VSMC growth, because it inhibits serum- or PDGF-mediated proliferation at a low cell density and potentiates it at a high cell density.\(^14,15\) PDGF and epidermal growth factor elevate the expression of TGF-\( \beta_1 \), which can also induce its own message.\(^16,17\) TGF-\( \beta_1 \) mRNA has been demonstrated recently to increase in aortas of experimental deoxycorticosterone acetate–salt hypertensive rats.\(^18\) These findings support the in vivo implication of synthesis and release of growth factors from VSMCs and their autocrine/paracrine role. However, the involvement of TGF-\( \beta_1 \) in essential hypertension has not yet been studied. The present investigation

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Supported by a grant from the Medical Research Council of Canada (MA-10803). V.H. was supported by a fellowship from Server Canada Inc.; U.K. was the recipient of a studentship from the Medical Research Council of Canada; and J.T. is a scholar of Fonds de la Recherche en Santé du Québec.

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focused on the growth of cultured VSMCs from SHRs and WKY rats at different cell densities in relation to contact inhibition and the expression of the proto-oncogene c-fos and TGF-β, as well as the effect of this growth factor added exogenously.

Methods

Cell Culture

Cultured VSMCs were obtained by an explant method from aortas of 10- to 13-week-old male SHRs and WKY rats (Charles River Canada, St. Constant, Quebec, or Taconic Farms Inc., Germantown, N.Y.), and their phenotypes in subculture were characterized as described previously. Briefly, cells from both origins that stained positively for smooth muscle-specific myosin antibodies presented at confluency a "hill-and-valley" formation, typical for smooth muscle cells in culture, and were of similar size. The cells were used for experiments between the sixth and 20th passages. When indicated, the cells were made quiescent after 16–20 hours of attachment by replacing the culture medium (Dulbecco's modified Eagle's medium [DMEM], 10% calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin) with defined serum-free medium (DMEM containing 2.5 μg/ml insulin, 2.5 μg/ml transferrin, and 2.5 ng/ml selenium) or DMEM plus 0.2% calf serum for 72 hours.

RNA Extraction and Northern Blot Analysis

Total cellular RNA was isolated from VSMCs by the acid guanidium thiocyanate–phenol–chloroform method. Ten micrograms of total RNA was denatured by heating (65°C for 10 minutes) in a buffer of 20 mM 3-(N-morpholino)propanesulfonic acid, pH 7.0, 50% formamide, and 1.8% formaldehyde gels in 20 mM 3-(N-morpholino)propanesulfonic acid, pH 7.0, 50% formamide, and 1.8% formaldehyde gels. The RNA was size-fractionated by electrophoresis on 1% agarose gel simultaneously in identical conditions. The gels were stained with ethidium bromide and were photographed and transferred to nylon filters (GeneScreen, New England Nuclear, Boston) in 3.0 M NaCl and 300 mM sodium citrate. The blots were prehybridized at 42°C for 3–4 hours in a buffer containing 50% formamide, 750 mM NaCl, 250 mM NaPO₄, 5 mM EDTA, 0.5% sodium dodecyl sulfate (SDS), 5 × Denhardt's solution (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), and 200 μg/ml sheared and denatured salmon sperm DNA. The blots were hybridized overnight at 42°C in 50% formamide, 1 × Denhardt's solution, 750 mM NaCl, 250 mM NaPO₄, 5 mM EDTA, 0.5% SDS, 10% dextran, and 200 μg/ml sheared and denatured salmon sperm DNA with phosphorothioate-32–labeled cDNA probes of v-fos or TGF-β. The v-fos probe consisted of the 5.75 kb HindIII fragment of pFBJ-2 plasmid. The TGF-β probe consisted of the 1.6 kb EcoRI fragment of pMurβ-2 plasmid. cDNA was radiolabeled by the random priming technique. After hybridization, the filters were washed with 50% formamide, 500 mM NaCl, 25 mM NaPO₄, 1 mM EDTA, and 0.5% SDS at 42°C for 30 minutes and with 75 mM NaCl, 5 mM NaPO₄, 1 mM EDTA, and 0.1% SDS at 65°C for 1 hour. They were exposed to Kodak X-ARPM1 film with two intensifying screens at −80°C. The developed films were scanned with a densitometer (Bio-Rad Laboratories, Mississauga, Ontario, Canada); areas under each peak, evaluated as optical density (OD) multiplied by peak width, were used as quantitative estimates of the amount of mRNA accumulation. The samples obtained from WKY rat and SHR VSMCs were prepared and run on the same gel simultaneously in identical conditions.

Thymidine Incorporation

[3H]Thymidine incorporation into newly synthesized DNA was performed as described previously. Briefly, quiescent VSMCs in 24-well cluster dishes were treated for 24 hours with TGF-β, from porcine platelets of at least 95% purity (R&D Systems, Inc., Minneapolis, Minn.) in DMEM plus 0.2% calf serum. The medium then was removed, and DMEM containing 0.5 μCi/ml [3H]thymidine (Du Pont, Montreal) was added for 2 hours. Each well was washed with 150 mM NaCl, fixed in ethanol/acetic acid (3:1) for 10 minutes, and washed with H₂O. Acid-insoluble material was precipitated by 15 minutes of incubation with cold 0.5 N perchloric acid, was washed, and DNA was extracted into 1.5 ml perchloric acid by heating at 80°C for 20 minutes. The radioactivity incorporated into newly synthesized DNA was determined in a liquid scintillation spectrometer.

Statistical Analyses

Values are given as mean ± SD. The level of significance of difference between means was evaluated by Students' t test for unpaired data and by three-way analysis of variance (ANOVA).

Results

Because our previously reported results have demonstrated an increased response to growth factors and an abnormality in G₁–S phase transition of the cell cycle, it seemed warranted to evaluate the expression of the proto-oncogenes implicated in the regulation of the G₁ phase (Figure 1). Cells were inoculated at 4 × 10⁵ cells/cm² and 17 × 10⁵ cells/cm² and left in 10% calf serum for 20 and 72 hours, respectively. The former cells remained at low cell density, whereas the latter reached high cell density of 70 to 100 × 10⁵ cells/cm², as can be seen in the experiment presented in Figure 2 (upper panel). In both cases, after 20 or 72 hours in 10% calf serum, cells were made quiescent by 72 hours of incubation in defined serum-free medium. Figure 1 follows the c-fos mRNA accumulation in response to 10% calf serum in quiescent WKY rat and SHR VSMCs at low and high inoculation densities. The proto-oncogene c-fos was rapidly induced from 15 minutes after
mitogenic stimulation, with a maximum at 30 minutes and a decline after 1 hour. No significant difference between WKY rat and SHR cells was seen at a low cell density. When the mRNA was quantified by densitometry in five different experiments, maximum expression was always observed at 30 minutes, and was 9.8±2.6 and 9.9±2.2 (OD×mm) versus 5.3±2.5 and 4.9±2.6 (OD×mm) at 15 minutes for WKY rat and SHR cells, respectively. At a high cell density, the c-fos expression at 15 minutes of calf serum stimulation was 8.4-fold higher in SHR when compared with WKY cells, as evaluated by densitometry in two independent experiments. A lesser 1.4-fold difference persisted at 30 minutes after the addition of growth stimuli. This increased expression of c-fos at a high cell density in VSMCs from SHRs seems to be a marker of reduced contact inhibition and hyper-responsiveness to mitogens.

To further investigate the effect of cell density on rat VSMC proliferation, cells were inoculated at low (1.8×10^5), medium (4×10^5), and high (100×10^5 cells/cm^2) densities, and their proliferation was followed in 10% calf serum by counting the cells over 3 days (Figure 2). WKY and SHR cells proliferated with significantly different kinetics (p<0.001, strain comparison by three-way ANOVA). Proliferative kinetics of VSMCs from both strains was significantly changed by cell density of inoculation (p<0.001, effect of cell density by three-way ANOVA). As seen in Figure 2, the difference between WKY and SHR cell number reached at 3 days increased from low to high density. At high seeding density, there was full growth arrest in cells of WKY origin, whereas proliferation continued over the following 3 days in cells derived from SHRs. We suggest that this lack of contact inhibition at a high density represents an additional intermediate phenotype of VSMCs in SHRs.

To search for potential regulators of the pathogenesis of this phenotype, we evaluated the expression and effect of TGF-β1. Cells were inoculated at the same densities as in experiments depicted in Figure 2. TGF-β1 mRNA accumulation was evaluated in cells growing in DMEM plus 10% calf serum at densities of 18×10^5 (low), 40×10^5 (medium), and 100×10^5 (high) cells/cm^2 in 25-cm^2 flasks. They were trypsinized 24, 48, and 72 hours later, and the cell number was determined in a Coulter counter. Data are expressed as mean±SD from two experiments performed in triplicate. ○, Wistar-Kyoto rats; ●, spontaneously hypertensive rats.

Figure 1. Expression of c-fos in vascular smooth muscle cells from Wistar-Kyoto (W) and spontaneously hypertensive (S) rats in response to calf serum stimulation. Low cell density: Cells were inoculated in DMEM plus 10% calf serum at 4×10^5 cells/cm^2 in 200-cm^2 flasks. After 20 hours of attachment, the medium was changed for defined serum-free medium for 72 hours. High cell density: Cells were inoculated at 17×10^3 cells/cm^2 and left in DMEM plus 10% calf serum for 3 days. Medium then was changed for defined serum-free medium for an additional 72 hours. Quiescent cells were stimulated in both cases by 10% calf serum, harvested (at 0, 15, 30, and 60 minutes), and RNA was extracted. Ten micrograms of total RNA was applied in each well. Gels were stained with ethidium bromide to control quantification of RNA samples. Membranes were hybridized with a probe of v-fos labeled with 32P by random priming.

Figure 2. Line graphs show growth of vascular smooth muscle cells from Wistar-Kyoto and spontaneously hypertensive rat at three different cell densities. Cells were inoculated in DMEM plus 10% calf serum at densities of 18×10^5 (low), 40×10^5 (medium), and 100×10^5 (high) cells/cm^2 in 25-cm^2 flasks. They were trypsinized 24, 48, and 72 hours later, and the cell number was determined in a Coulter counter. Data are expressed as mean±SD from two experiments performed in triplicate. ○, Wistar-Kyoto rats; ●, spontaneously hypertensive rats.
potential role of TGF-β₁ in the altered contact inhibition in SHR cells. The presence and secretion of TGF-β₁ protein currently are being investigated in our laboratory. Because TGF-β₁ enhances its own expression, it is conceivable that this positive autocrine/paracrine effect is exaggerated in VSMCs from SHRs.

The DNA synthesis in response to exogenous TGF-β₁ was studied at low and high cell densities. VSMCs were synchronized for 3 days in DMEM plus 0.2% calf serum and then treated with increasing concentrations of TGF-β₁ in DMEM plus 0.2% calf serum for 24 hours (Figure 4). In these experiments, control VSMCs from WKY rats and SHRs responded to 10% calf serum stimulation with significant differences at low densities (WKY, 20,500±1,800 cpm/well; SHR, 30,100±10,000 cpm/well, *p*<0.001, WKY versus SHR by Student's *t* test for unpaired data) and high densities (WKY, 79,400±10,000 cpm/well; SHR, 96,000±3,300 cpm/well, *p*<0.02, WKY versus SHR by Student's *t* test for unpaired data), as shown previously.⁶ The dose response to exogenous TGF-β₁ is illustrated in Figure 4. SHR cells presented higher basal [³H]thymidine incorporation at both cell densities, which was not seen in our previous studies.⁷ This most probably was due to cell synchronization in DMEM plus 0.2% calf serum in contrast to our previous use of DMEM plus plasma-derived serum or defined serum-free medium. Because TGF-β₁ has been shown to need a minimal presence of PDGF for its effect,⁸ 0.2% calf serum was used here, and the basal levels of [³H]thymidine incorporation reflected a described hyperresponsiveness of VSMCs from SHRs to growth factors. The data revealed no dose effect of TGF-β₁ at a low density in both WKY and SHR VSMCs. At a high cell density, there was no effect of TGF-β₁ in WKY VSMCs. In contrast, in cells of SHR origin, [³H]thymidine incorporation increased significantly with escalating concentrations of TGF-β₁ (*p*<0.001 by three-way ANOVA).

Additional experiments examined the density-dependent rise in [³H]thymidine incorporation stimulated by TGF-β₁ in SHRs. Figure 5 depicts the effect of increasing cell density on the response to 10 ng/ml TGF-β₁. At a low density, there was no response to TGF-β₁ in either WKY or SHR cells. An optimal response was observed at a medium cell density with also a slight effect of TGF-β₁ in WKY cells and a greatest difference between SHR and WKY cells. The difference persisted at a high density, at which TGF-β₁ again had no effect on WKY cells. Given the fact that cells in these experiments were synchronized before TGF-β₁ addition, we did not observe the inhibitory effect of TGF-β₁ at low cell densities in either WKY or SHR cells, as has been demonstrated in growing VSMCs.¹⁵,²⁶

**Discussion**

The growth-promoting effect of TGF-β₁ was proposed to be responsible for the establishment and maintenance of the multilayered “hill” formation in confluent VSMCs in vitro.¹⁴ The “hill-and-valley” for-
mations occurred earlier in VSMCs from SHRs. An abnormal expression of TGF-β1 mRNA in cells of SHR origin suggests that this growth factor may be secreted in higher amounts and modulate the differential proliferative characteristics of SHR cells in an autocrine/paracrine manner. It has been shown that TGF-β1 positively regulates its own expression. The data presented here demonstrate an abnormal expression and response to TGF-β1 and suggest that the autoinduction of TGF-β1 is particularly enhanced in growing VSMCs from SHRs. Such a defect in feedback regulation of an endogenous growth factor may be involved in the expression of abnormal growth phenotypes in SHRs, resulting in greater VSMC proliferation and increased peripheral vascular resistance. Nevertheless, the pathogenetic involvement of the increased expression and effect of TGF-β1 in primary hypertension requires genetic studies of segregation of this phenotype with high blood pressure. TGF-β1 also induces the expression of PDGF-A mRNA in VSMCs and increases the secretion of PDGF-like protein. Whether or not TGF-β1 exerts its growth stimulatory effect directly or indirectly via PDGF-like molecules is not yet clear. Investigation with neutralizing antibody and antisense RNA of these growth factors is underway in our laboratory.

Acknowledgments

We express our gratitude to Carole Long for her technical assistance, to Louise Chervetts for her excellent secretarial work, and to Ovid Da Silva for editing this manuscript.

References


FIGURE 5. Bar graph shows effect of cell density on transforming growth factor β1 (TGF-β1)-stimulated [3H]thymidine incorporation into DNA of vascular smooth muscle cells from Wistar-Kyoto (WKY) rats and spontaneously hypertensive rats (SHRs). Cells were inoculated in 24-well cluster dishes at low, medium, or high densities in DMEM plus 10% calf serum. After 20 hours of attachment, the medium was replaced by DMEM plus 0.2% calf serum for 72 hours. Number of quiescent cells was determined in control wells (in 10^4 cells/cm^2): 1, 1±2; 2, 1±2; 3, 3±4; 4, 24±1; 5, 58±3; 6, 57±7; 7, 44±5; 8, 60±6; 9, 111±18; 10, 537±25; 11, 239±9; 12, 305±15; mean±SD, n=4. Quiescent cells were treated with 10 ng/ml TGF-β1 in DMEM plus 0.2% calf serum for 24 hours. Cells then were pulsed by [3H]thymidine for 2 hours, and the radioactivity incorporated was measured in precipitated acid-insoluble material. Net effect of TGF-β1 was calculated as counts per minute per well of 10 ng TGF-β1-stimulated cells minus counts per minute per well of nonstimulated, quiescent cells. *p<0.001 by Student's t test, WKY rats vs. SHR.

**KEY WORDS** • vascular smooth muscle • proto-oncogenes • growth substances • contact inhibition • essential hypertension • spontaneously hypertensive rats
Transforming growth factor beta 1 expression and effect in aortic smooth muscle cells from spontaneously hypertensive rats.
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_Hypertension_. 1991;17:896-901
doi: 10.1161/01.HYP.17.6.896

_Hypertension_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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