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Osteopontin Deficiency in Rat Vascular Smooth Muscle Cells is Associated with an Inability to Adhere to Collagen and Increased Apoptosis

Andrea S. Weintraub, Lynn M. Schnapp, Xinjie Lin, and Mark B. Taubman

Department of Pediatrics (ASW, XL), Division of Pulmonary Medicine (LMS) and The Cardiovascular Institute (MBT), Department of Medicine (LMS, MBT), The Mount Sinai School of Medicine, New York, New York.

SUMMARY: Osteopontin (OPN) is an extracellular matrix protein that has been implicated in vascular smooth muscle cell (VSMC) adhesion. We have previously described the generation of OPN-deficient VSMC that displayed altered adhesion to collagen. We have examined further the causes and consequences of this altered adhesion. OPN-deficiency was associated with a significant reduction in surface expression of α1 and β1 integrins (mean fluorescence intensity α1: OPN-deficient 0.135 $\pm$ 0.04 vs. control 0.313 $\pm$ 0.05, $p < 0.0001$; β1: OPN-deficient 0.398 $\pm$ 0.09 vs. control 0.570 $\pm$ 0.05, $p < 0.004$). Treatment of normal VSMC with antibody to α1 recapitulated the adhesion defect. OPN-deficient cells without collagen exposure had an apoptotic fraction of 1.9%, which increased to 95.7% after 24 hours exposure to collagen. Exogenous OPN added to cultures within 15 minutes of plating restored normal cell adhesion, but did not prevent cells from undergoing apoptosis. Normal VSMC had no detectable apoptosis after 24 hours incubation in suspension, whereas OPN-deficient cells had an apoptotic fraction of 37.5% when incubated in suspension under the same conditions. The data suggest that OPN-deficient VSMC have two distinct abnormalities: an α1β1-mediated inability to adhere normally to collagen and an increased propensity for apoptosis. (*Lab Invest 2000, 80:1603–1615*).

Osteopontin (OPN) is a secreted extracellular matrix (ECM) glycoprotein with an arginine-glycine-aspartate (RGD) binding motif previously shown to interact with multiple integrin subunits (Bayless et al, 1998; Denda et al, 1998; Liaw et al, 1995b; Smith and Giachelli, 1998; Smith et al, 1996). Although originally isolated from bone, OPN has been identified in many tissues, including vascular smooth muscle cells (VSMC) (Brown et al, 1992; Chambers et al, 1992; Giachelli et al, 1993; 1994; Green et al, 1995; Nomura et al, 1988; Shanahan et al, 1993). OPN mRNA expression in cultured rat VSMC is induced by mediators of early gene activation and by arterial injury (Gadeau et al, 1993; Giachelli et al, 1993; Green et al, 1995; Shanahan et al, 1993; Wang et al, 1996). In the uninjured rat arterial wall, medial VSMC express low levels of OPN mRNA and protein (Giachelli et al, 1993). After balloon injury, OPN mRNA and protein levels increase in endothelial cells and in medial and neointimal VSMC (Giachelli et al, 1993; Liaw et al, 1995a). OPN is also a prominent component of human atherosclerotic plaques, where it is associated with macrophage-derived foam cells and areas of calcification (Fitzpatrick, 1996; Hirota et al, 1993; Ikeda et al, 1993; Liaw et al, 1995a; O'Brien et al, 1994; Shanahan et al, 1994).

αvβ1, αvβ3, and αvβ5 integrins act as OPN receptors and mediate VSMC adhesion, spreading, and migration in vitro and in vivo (Liaw et al, 1995a, 1995b; Yue et al, 1994). α4β1 promotes leukocyte adhesion to OPN, which may be important for cellular responses during tissue injury (Bayless et al, 1998). Ligation of α8β1 by OPN may be important in the regulation of epithelial-mesenchymal interactions during renal morphogenesis (Denda et al, 1998). OPN promotes adhesion of cultured aortic endothelial and VSMC and is chemotactic for VSMC (Liaw et al, 1994; Yue et al, 1994). OPN is expressed by human trophoblasts (Daiter et al, 1996; Gabinskaya et al, 1998) during placental vascular remodeling and may promote cell migration during gastrulation (Thayer et al, 1995). OPN mRNA is increased in *ras*-transformed NIH 3T3 cells and correlates with levels of *ras*-expression and metastatic ability (Chambers et al, 1992). Transfection of tumor cell lines with OPN increases their malignant phenotype (Denhardt and Guo, 1993; Weber et al, 1996, 1997). Conversely, *ras*-transformed fibroblasts transfected with OPN-antisense constructs have decreased malignant potential (Behrend et al, 1994; Weber et al, 1997).

We have recently reported the generation of rat aortic VSMC clones that stably underexpress OPN antisense mRNA (Weintraub et al, 1996). These clones, which have an approximately 60% reduction in OPN secretion, exhibited the typical spindle shaped morphology of normal VSMC and displayed identical

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Address reprint requests to: Dr. Andrea S. Weintraub, Box 1508, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029. Fax: 212 534 5207; Email: andrea.weintraub@mssm.edu

growth rates on plastic monolayers. When cultured on 3D collagen gels, the majority of OPN-deficient VSMC did not adhere, spread, or invade collagen matrices. OPN-deficient cells that did succeed in adhering to the gels did not grow. These defects were corrected by the addition of exogenous OPN to the culture medium. In the current study, we have used these clones to examine further the consequences of the OPN-sensitive impairment of VSMC adhesion. We now report that OPN-deficiency is associated with decreased surface expression of the collagen receptor $\alpha1\beta1$, and that the nonadhesive phenotype can be recapitulated by the treatment of normal VSMC with antibody against $\alpha1\beta1$. Moreover, prolonged exposure of OPN-deficient VSMC to native collagen results in growth arrest and the rapid induction of apoptosis.

Results

OPN-Sensitive Impairment of VSMC Adhesion

We have previously demonstrated that the majority of OPN-deficient VSMC cultured on collagen gels did not adhere, spread, or invade the matrices, and that addition of OPN to the cultures at the time of plating onto collagen corrects the adhesion defect (Weintraub et al, 1996). To determine the window in which OPN can rescue adhesion, OPN-deficient cells were treated with 400 ng/ml of OPN at the time of plating onto collagen or at various times after plating (Fig. 1). Addition of OPN at the time of plating or 15 minutes after plating on collagen rescued the adhesion defect; rescue was not seen when OPN was added to the cultures at 30 or 60 minutes. The rapid time course suggests that normal cell adhesion requires the presence of OPN and that de novo OPN synthesis cannot rescue.

To further determine whether new protein synthesis is required for cell adhesion, normal VSMC were pretreated with 10 μM cycloheximide prior to plating onto collagen gels (Table 1). The percentage of cells adherent to the collagen gels 1 hour and 3 hours after plating was unaffected by treatment with cycloheximide, suggesting that de novo protein synthesis is not necessary for normal adhesion.

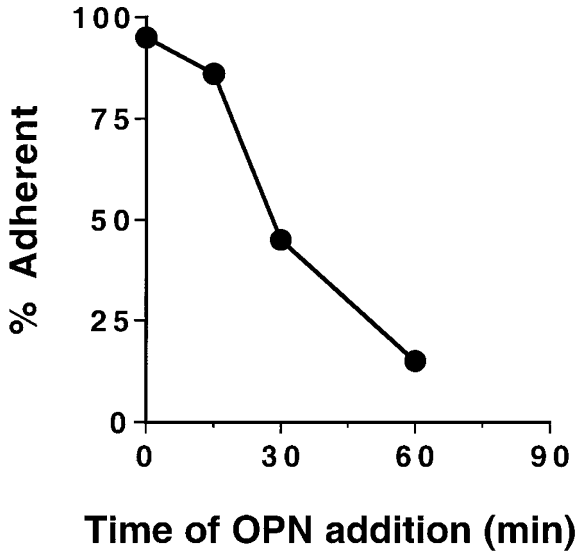

Figure 1.

The effect of exogenous OPN on cell adhesion. Osteopontin (OPN) deficient vascular smooth muscle cells (VSMC) were plated onto collagen gels at 50% confluence. Purified rat OPN (400 ng/ml) was added to the cultures at the time of plating or at the times indicated after plating. After incubation for 24 hours at 37° C, the culture medium was collected, the gels were rinsed with phosphate buffered saline (PBS), and cell counts on the combined medium and washes were determined. % Adherent [(number of cells plated − number of cells in culture medium) ÷ number of cells plated] is plotted as a function of the time after OPN addition to the cultures.

OPN-Deficient VSMC Have Decreased Expression of $\alpha1\beta1$ Integrin

The ability of OPN-deficient cells to adhere to collagen was not dependent on plating density (Fig. 2). To determine the mechanism underlying OPN-mediated cell adhesion, VSMC surface integrin expression was examined by indirect immunofluorescence. $\alpha1\beta1$ and $\alpha2\beta1$ are receptors for native Type I collagen (Montgomery et al, 1994); $\alpha v\beta3$ is a receptor for OPN, vitronectin, and other proteins (Heino, 1996; Liaw et al, 1995b); and $\alpha5\beta1$ is a fibronectin receptor (Zhang et al, 1995). The integrin profile of VSMC clones is shown in Figure 3. The mean fluorescence intensities (MFI) for OPN-deficient clones AS 2/7, AS 2/28, AS 2/22, and AS-1 2/22 (△) are presented on the right. As controls, MFI for empty vector clones XH, XH 2/7, and XH 2/14 (●) are shown in the left column. Also included as controls in the left-hand column of each panel are the MFI for replicate experiments with normal VSMC (◩) and VSMC clone AS 2/2 (◗). This OPN antisense-infected clone did not express significant levels of OPN antisense mRNA and had normal secreted OPN levels (Weintraub et al, 1996). There was a significant reduction in the expression of $\alpha1$ and $\beta1$ in the OPN-deficient VSMC compared with the controls (MFI $\alpha1$: OPN-deficient 0.135 ± 0.04 vs. Control 0.313 ± 0.05, Fisher's protected least significance difference (PLSD) $p < 0.0001$; $\beta1$: OPN-deficient 0.398 ± 0.09 vs. Control 0.570 ± 0.05, Fisher's PLSD $p < 0.004$).

Table 1. Role of De Novo Protein Synthesis in VSMC Adhesion

	% Adherent‡	
	1 hour	3 hours
Control*	99%	95%
Cycloheximide* (0 time)	99%	98%
Cycloheximide§ (1 hour preincubation)	97%	95%
Cycloheximide§ (3 hours preincubation)	95%	94%

* Vascular smooth muscle cell (VSMC) cultures grown on plastic monolayers were trypsinized and plated on collagen in the absence (Control) or presence (0 time) of 10 μM cycloheximide.

§ Duplicate cultures were grown on plastic monolayers in the presence of 10 μM cycloheximide. At the times indicated, cells were trypsinized and plated on collagen gels at a density of 5×10^4 cells/well (50% confluent).

‡ The culture medium was collected 1 hour and 3 hours after plating and cell number was determined. % Adherent = (number of cells plated − number of cells in culture medium) ÷ number of cells plated.

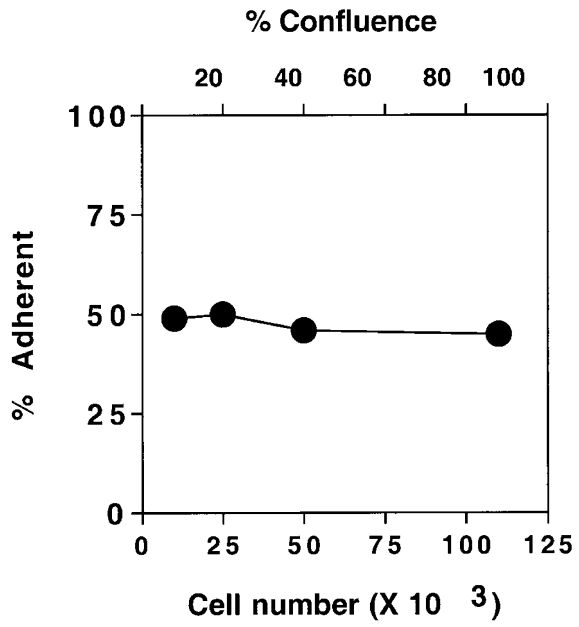

Figure 2.
Effect of cell density on adhesion of OPN-deficient cells to collagen. OPN-deficient VSMC were plated onto collagen gels at varying densities. After incubation for 30 minutes at 37° C, the culture medium was collected, the gels were rinsed with PBS, and cell counts on the combined medium and washes were determined. % Adherent [(number of cells plated − number of cells in combined medium and washes) ÷ number of cells plated] is plotted as a function of the number of cells plated (lower *x* axis). Percent (%) confluence is shown in the upper *x* axis.

There were no significant differences in the expression of $\alpha 2$, $\alpha 5$, or $\beta 3$ between OPN-deficient and control VSMC ($\alpha 2$: OPN-deficient 0.03 ± 0.03 vs. Control 0.04 ± 0.05, NS; $\alpha 5$: OPN-deficient 0.351 ± 0.06 vs. Control 0.473 ± 0.08, NS; $\beta 3$: OPN-deficient 0.024 ± 0.02 vs. Control 0.051 ± 0.06, NS). This suggests that the OPN-sensitive adhesion defect seen on collagen is caused by decreased surface expression of the $\alpha 1\beta 1$ collagen receptor.

mAb Directed Against $\alpha 1\beta 1$ Recapitulates the OPN-Deficient VSMC Phenotype on Collagen Gels

To examine further the role of $\alpha 1\beta 1$ in mediating VSMC adhesion to collagen, normal VSMC were incubated in serum-free Dulbecco's modified Eagle medium (DMEM) with function-blocking mAb to $\alpha 1$ at a saturating concentration of 100 μg/ml, and plated onto collagen gels. VSMC adhesion was blocked by $\alpha 1$ mAb (Fig. 4A), but was unaffected by incubation with isotype-specific nonimmune IgG (Fig. 4B) or 100 μg/ml $\alpha 5$ mAb (not shown). Cells that remained adherent to the gels after treatment with $\alpha 1$ mAb failed to grow in the presence of serum and remained as isolated, spherical cells after 72 hours incubation. This effect was identical to the phenotype seen when OPN-deficient VSMC were plated onto collagen gels (Fig. 4C) (Weintraub et al, 1996).

$\alpha 1$ mRNA is Decreased in OPN-Deficient VSMC and is Regulated by Exogenous OPN

By RNA blot analysis, quiescent (ie, incubation in defined medium for 48 hours) normal VSMC and VSMC infected with the empty retroviral vector expressed abundant $\alpha 1$ mRNA at baseline, whereas OPN-deficient VSMC expressed minimal amounts (Fig. 5). To determine whether OPN directly regulates $\alpha 1$ synthesis, RNA was harvested from VSMC treated with 800 ng/ml of recombinant OPN. In normal cells, $\alpha 1$ mRNA expression increased 24 to 72 hours after OPN treatment (Fig. 5A). $\alpha 1$ mRNA expression in OPN-deficient cells increased 2 hours after OPN treatment, peaked at 48 hours and returned toward baseline at 72 hours (Fig. 5B). These findings were reproducible in triplicate experiments.

OPN-Deficient VSMC Display Abnormal Growth Following Collagen Exposure

To determine the consequences of abnormal cell adhesion, OPN-deficient VSMC grown to confluence on plastic culture plates were trypsinized, suspended in DMEM supplemented with 10% calf serum (CS), and then incubated on collagen gels for 15, 30, or 60 minutes. Nonadherent cells in the culture medium overlying the gels were collected and replated on plastic (Fig. 6, open shapes). As a comparison, duplicate cultures of OPN-deficient cells were trypsinized and incubated in suspension at 37° C in conical tubes, without exposure to collagen, before replating on plastic (Fig. 6, closed shapes). Trypan blue staining demonstrated that 100% of cells were intact immediately after collagen exposure. After a 15-minute exposure to collagen, the growth of OPN-deficient cells on plastic was comparable with that of OPN-deficient cells that were not collagen-exposed. In contrast, OPN-deficient cells with longer collagen exposures did not grow after replating onto plastic, whereas those not exposed to collagen grew normally. Wild-type VSMC incubated on collagen gels for 30 minutes, released from the gels using Type II collagenase, and then replated onto plastic, also grew normally (Fig. 6, hatched circle).

OPN-Deficient VSMC Undergo Apoptosis After Continuous Collagen Exposure

OPN-deficient VSMC grown on plastic were trypsinized, suspended in DMEM/10% CS, and incubated on collagen gels. Nonadherent cells were fixed in ethanol, stained with propidium iodide (PI), and analyzed for cell cycle distribution using flow cytometry (Fig. 7 and Table 2). Freshly trypsinized OPN-deficient VSMC without collagen exposure had 1.9% apoptotic cells (0 time). After continuous exposure to collagen, the apoptotic fraction was 1.9% at 15 minutes, 1.1% at 30 minutes, 7.7% at 1 hour, 28.1% at 4 hours, 38.5% at 12 hours, and 95.7% at 24 hours. The percent decrease in the G_0-G_1 and S phase fractions was comparable with the percent increase in the apoptotic fraction.

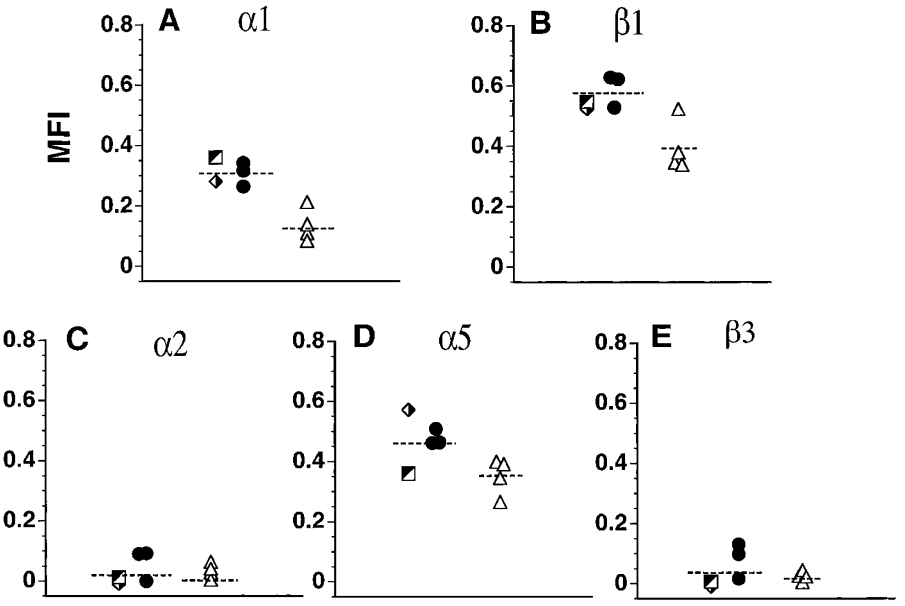

Figure 3.

Integrin profile of VSMC clones. Surface integrin expression of VSMC clones was evaluated by indirect immunofluorescence using flow cytometry. Normalized mean fluorescence intensities (MFI) at 488 nm are shown on the *y* axis and represent the average of 4 replicate experiments. MFIs for OPN-deficient clones (△) are presented in the right-hand column. MFIs for controls are presented on the left and include: empty vector clones (●), normal VMSC (◩), and VSMC clone AS 2/2 (◆). AS 2/2 failed to express high levels of OPN antisense mRNA and did not inhibit secreted OPN levels. *A*, α1; *B*, β1; *C*, α2; *D*, α5; *E*, β3. (——) represents the average MFI for either the OPN-deficient clones (△) or the controls (●, ◩, ◆).

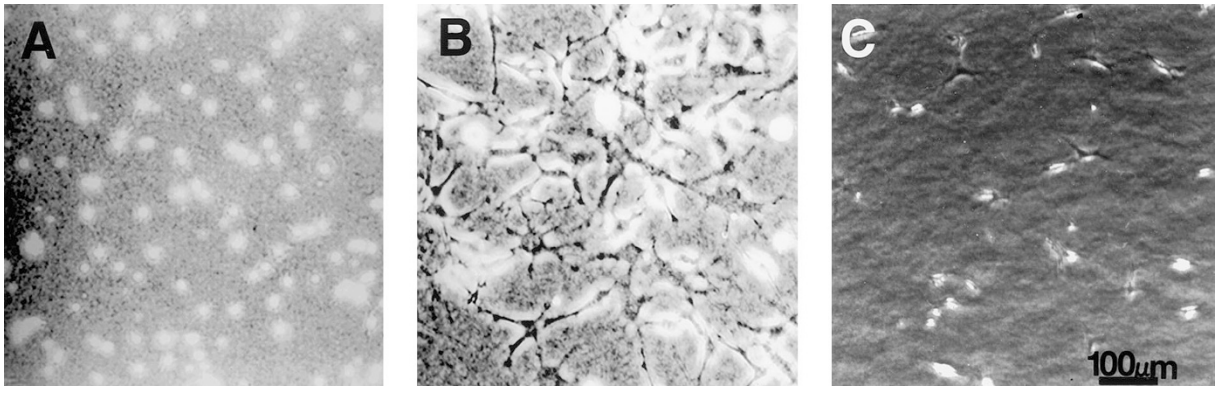

Figure 4.

Effect of mAb to α1 on VSMC adhesion to collagen. Normal VSMC were plated in triplicate at a density of 5×10^4 on collagen gels in 12-well dishes in the presence of isotype-specific nonimmune IgG (*B*) or 100 μg/ml of mAb directed against α1 (*A*). OPN-deficient VSMC plated at a density of 5×10^4 on collagen gels in 12-well dishes are shown for comparison (*C*). Representative photographs corresponding to planes within the gel matrix were taken 72 hours after plating.

To confirm that the sub-G_0 cell fraction was apoptotic, OPN-deficient VSMC were also examined by phase contrast microscopy after in situ detection of DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). After 1 hour of collagen exposure, nuclei of the nonadherent, OPN-deficient VSMC displayed strong TUNEL-positive staining (Fig. 8A). No staining was seen in OPN-deficient cells suspended in DMEM/10% CS without collagen exposure (Fig. 8B).

To determine the duration of collagen exposure required for initiation of the apoptotic program, OPN-deficient VSMC grown to confluence on plastic culture plates were trypsinized, suspended in DMEM/10% CS, and incubated on collagen gels. Nonadherent cells recovered from the culture medium after 15, 30, or 60 minutes of collagen exposure were incubated in suspension for 24 hours in plastic conical tubes before cell cycle analysis was performed (Table 3). The apoptotic fraction was 6.9% after 15 minutes of collagen exposure and increased to 25% at 30 minutes and 78.4% at 60 minutes.

To determine whether OPN-deficient VSMC were more prone to apoptosis even in the absence of collagen exposure, OPN-deficient cells were freshly trypsinized and incubated in suspension at 37° C for 24 hours (Table 4). The apoptotic fraction was 37.5%. In contrast, normal VSMC and VSMC infected with the retroviral vector lacking an OPN antisense cDNA insert incubated in suspension under the same conditions

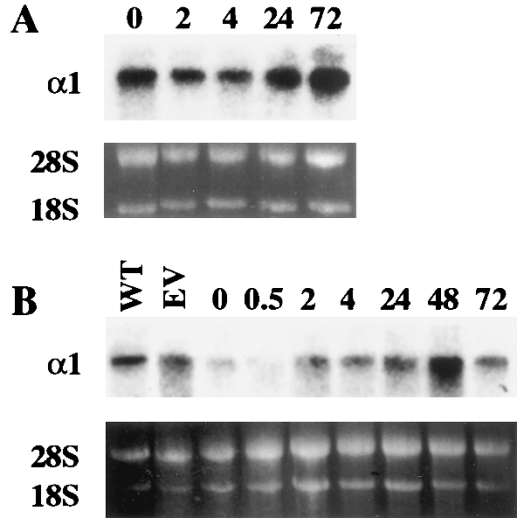

Figure 5.

RNA blot analysis of α1 expression in cultured VSMC. *A*, Total RNA (10 μg/lane) was isolated from unstimulated quiescent normal VSMC (Time 0) and after stimulation with exogenous OPN (800 ng/ml) for the indicated periods of time (hours). *B*, Total RNA was isolated from unstimulated quiescent normal cells (WT), VSMC infected with the empty retroviral vector (EV), and OPN-deficient VSMC stimulated with exogenous OPN (800 ng/ml) for the indicated periods of time (0–72 hr). RNA isolation was performed in triplicate and three replicate blots were hybridized with ^{32}P-labeled α1 probe as described in the "Materials and Methods" section. Equal loading of total RNA was verified by ethidium bromide staining of the 18S and 28S ribosomal RNA.

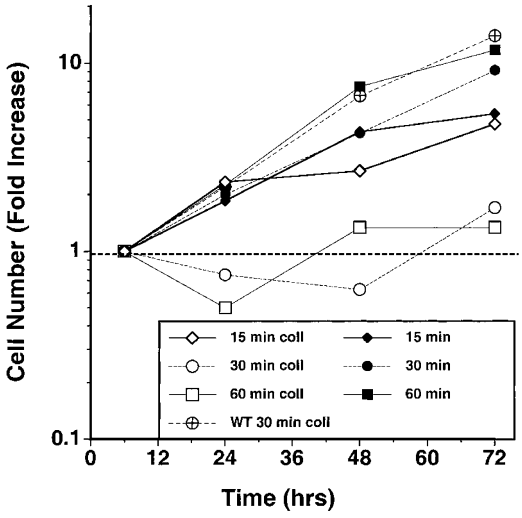

Figure 6.

Growth of OPN-deficient VSMC in monolayer after collagen exposure. OPN-deficient VSMC were plated on 12-well collagen gels at a density of 4×10^4 cells per gel. At 15, 30, or 60 minutes, nonadherent cells in the culture medium were replated onto plastic 12-well dishes (*open shapes*). Duplicate cultures of OPN-deficient cells were incubated in suspension in conical tubes, without exposure to collagen, before replating on plastic (*closed shapes*). Wild-type VSMC were also incubated on collagen gels for 30 minutes, released from the gels with Type II collagenase, and then replated on plastic (*hatched circle*). Cell counts were obtained in triplicate at 6, 24, 48, and 72 hours after replating onto plastic and plotted as the fold increase in cell number over time zero on a logarithmic scale.

had very low levels of apoptosis (0% and 4.4%, respectively). OPN-deficient VSMC grown to confluence on plastic culture plates were also trypsinized and replated onto plastic plates instead of collagen for 15 minutes. These cells were then released from the plates by EDTA and incubated in suspension for 24 hours. The apoptotic fraction was 21% (p = NS compared with cells incubated in suspension alone; $p < 0.005$ compared with cells exposed to collagen for 15 minutes, Table 3). This suggests that at baseline, OPN-deficient VSMC are more prone to undergo apoptosis. When OPN-deficient cells without collagen exposure were suspended in DMEM/10% CS/1.5% methylcellulose for 24 hours to prevent cell-cell contact, the apoptotic fraction remained high (24%, p = NS compared with cells grown in suspension without methylcellulose). The apoptotic fraction was also independent of cell density (Table 5).

As shown in Figure 1, early addition of OPN to collagen gels rescues the adhesion defect of OPN-deficient cells. Although addition of exogenous OPN to OPN-deficient cells at or 15 minutes after plating markedly increased adhesion on collagen gels, it had no significant effect on the percentage of apoptotic cells in the nonadherent fraction (Table 6). The apoptotic fraction of OPN-deficient cells adherent to the collagen gels (10.7%) was higher than in cells grown on plastic (1.9%), but lower than in cells grown in suspension (37.5%). This was not altered by the addition of OPN at 15 minutes (10.2%). The addition of OPN also did not significantly alter the percentage of apoptosis of OPN-deficient cells incubated in suspension in the absence of collagen exposure (49%, Table 4).

Discussion

OPN is a secreted, phosphorylated glycoprotein found in abundance in atherosclerotic plaques and in the injured arterial wall (Fitzpatrick et al, 1994; Giachelli et al, 1993; Ikeda et al, 1993; O'Brien et al, 1994). We have recently shown that when plated on collagen gels, OPN-deficient VSMC display an adhesion defect that can be rescued by the addition of exogenous OPN (Weintraub et al, 1996). In the current report, we demonstrate that OPN-deficiency in VSMC is associated with two intrinsic cell defects: an adhesion defect and an increased propensity to undergo apoptosis. OPN-deficiency is associated with decreased expression of the $\alpha1\beta1$ collagen receptor; the nonadhesive phenotype can be reproduced by treatment of normal VSMC with antibody against α1.

The integrin repertoire of VSMC is remarkable for the reciprocal expression of the $\alpha1\beta1$ and $\alpha2\beta1$ collagen/laminin receptors (Skinner et al, 1994). In our current study, we found that OPN deficiency (63 $\pm$ 2% reduction in secreted OPN compared with normal VSMC or clones containing the empty vector alone (Weintraub et al, 1996)) was associated with decreased expression of the $\alpha1\beta1$ collagen receptor (57% decrease in α1 and 43% decrease in β1) and a decrease in α1 mRNA expression. In addition, OPN directly regulated α1 mRNA expression. Of note, levels of α1 mRNA were increased in normal cells 72 hours after treatment with OPN. In contrast, levels of α1 mRNA were decreasing in OPN-deficient VSMC 72

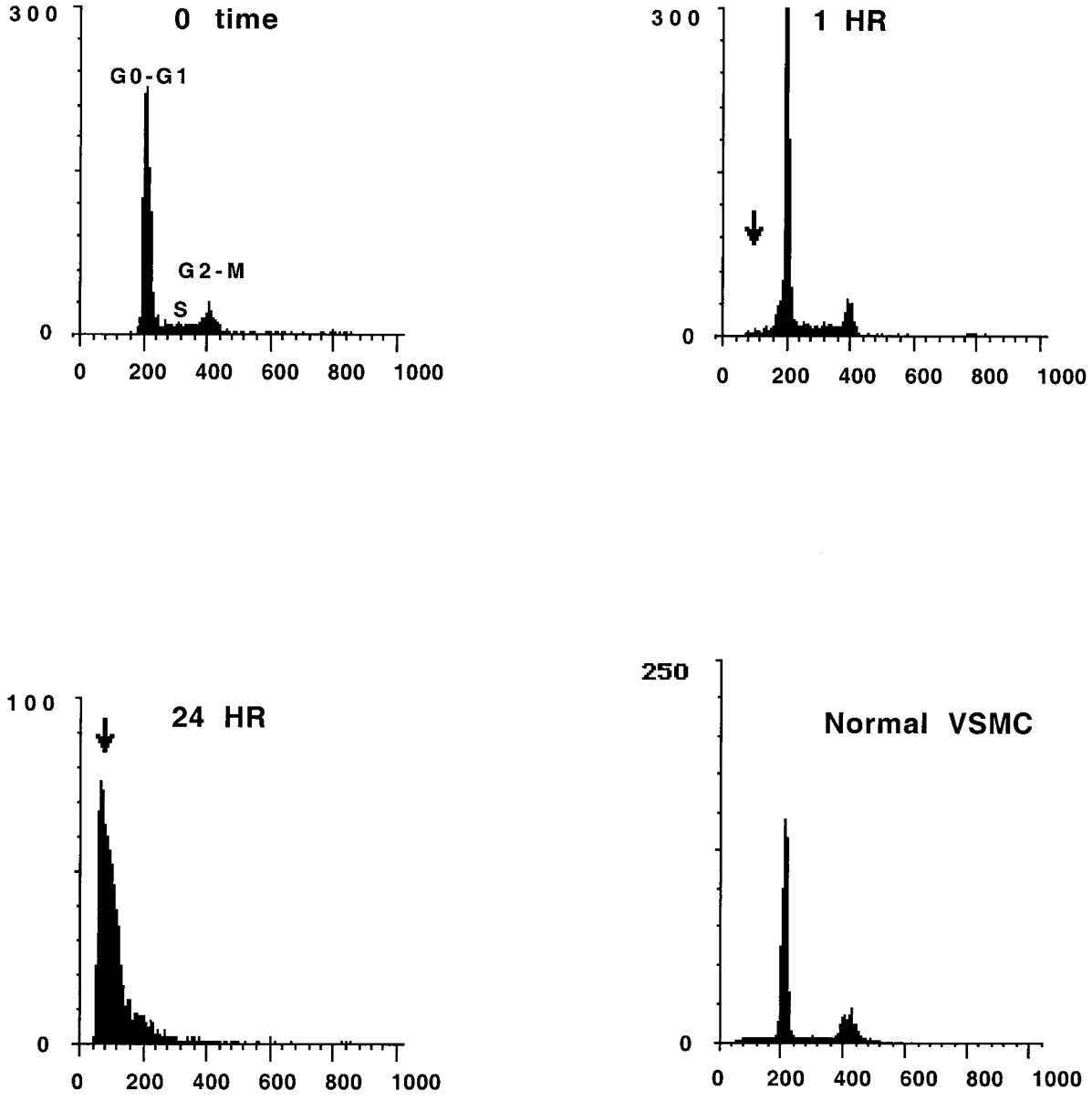

Figure 7.

OPN-deficient VSMC analyzed for DNA content. OPN-deficient VSMC were plated on 12-well collagen gels at a density of 5×10^4 cells per gel. Nonadherent cells were fixed and stained with propidium iodide (PI; 10 μg/ml)-RNAse A (0.5 mg/ml). Representative histograms of DNA content are shown (excitation at 488 nm with detection at 620–700 nm for 10,000 events per histogram). Each experiment was performed on at least three different occasions using two different OPN-deficient clones. Zero (0) time represents freshly trypsinized OPN-deficient cells suspended in DMEM/10% CS without exposure to collagen. Normal VSMC represents wild-type cells grown in monolayer on plastic without collagen exposure. *Arrows* indicate apoptotic cell fractions.

hours after OPN treatment. These data suggest that in normal cells, endogenous secretion of OPN may be sufficient to maintain relatively high levels of α1 mRNA at all times. In contrast, OPN-deficient cells do not produce enough OPN to maintain high levels of α1 mRNA; although exogenous OPN causes a rapid induction of mRNA, the increase cannot be sustained without addition of further OPN.

Because there is a lack of sequence homology between OPN and α1 (GenBank accession #124941) or β1 (GenBank accession #1352494) integrin subunits (Smith-Waterman scores 43 and 54, respectively), it is unlikely that the decrease in $\alpha 1\beta 1$ is caused by a direct effect of the antisense construct on α1 or β1 mRNA. Similarly, because VSMC infected with the empty retroviral vector expressed abundant α1 mRNA and protein, it is unlikely that the effect is an artifact of the infection and cloning process. The inability of the OPN-deficient VSMC to adhere normally to the collagen gels can be attributed in part to their relative $\alpha 1\beta 1$ deficiency, because this adhesion defect could be reproduced by the incubation of normal VSMC with antibodies against α1. As might be expected from this data, OPN-deficient cells do adhere and spread well on plastic or Matrigel (data not shown), which is a reconstituted basement membrane composed primarily of laminin. Adhesion to laminin is predominantly via $\alpha 3\beta 1$ or $\alpha 6\beta 1$ (Hynes and Bader, 1997; Li et al, 1994).

Table 2. Cell Cycle Distribution of OPN–Deficient VSMC During Continuous Collagen Exposure

	% APO	% G_0-G_1	% S	% G_2-M
0 time*	1.9	75.3	14.4	8.4
15 minutes§	1.9	72.1	16.7	9.3
30 minutes§	1.1	74.3	17	7.7
1 hour§	7.7	72.3	12.9	7.1
4 hours§	28.1	57.7	7.3	6.9
12 hours§	38.5	41.9	7.5	12.1
24 hours§	95.7	0	4.3	0
Normal VSMC‡	0	80.7	3.7	15.6

* OPN–deficient VSMC grown in monolayer on plastic plates were trypsinized and immediately fixed, stained with propidium iodide (PI), and analyzed for cell cycle distribution using flow cytometry.

§ OPN–deficient VSMC were trypsinized, suspended in DMEM/10% CS, and incubated on collagen gels. At the times indicated, nonadherent cells in the culture medium were fixed, stained with PI, and analyzed for cell cycle distribution.

‡ Normal VSMC grown in monolayer on plastic plates were trypsinized and immediately fixed, stained with PI, and analyzed for cell cycle distribution.

Table 3. Relationship Between Duration of Collagen Exposure and Apoptosis in OPN–Deficient VSMC

	% APO	% G_0-G_1	% S	% G_2-M
15 minutes coll*	6.9	21.9	56.2	15.5
30 minutes coll*	25	57.8	10.8	4.5
60 minutes coll*	78.4	12.3	5.6	1.8
24 hours coll*	100	0	0	0

* OPN–deficient VSMC were trypsinized, suspended in DMEM/10% CS, and incubated on collagen gels. At the times indicated, nonadherent cells in the culture medium were collected, centrifuged, resuspended in fresh DMEM/10% CS, and incubated for 24 hours at 37° C in plastic conical tubes. Cells were then centrifuged, washed with PBS, fixed, stained with PI, and analyzed for cell cycle distribution using flow cytometry.

The failure of OPN-deficient cells to adhere to collagen was accompanied by the rapid induction of apoptosis. There is considerable evidence to suggest that integrin-mediated cell adhesion to the ECM can influence growth, differentiation, and survival (Adams and Watt, 1993; Bates et al, 1994; Chen et al, 1997; Giancotti, 1997; Meredith et al, 1998; Montgomery et al, 1994; Re et al, 1994; Ruoslahti and Reed, 1994; Schoenwaelder and Burridge, 1999). Integrin-mediated cell-matrix interactions can activate intracellular signaling pathways (eg, focal adhesion kinase, protein kinase C, changes in intracellular pH, Ca^{2+} flux) that inhibit apoptosis (Damsky and Werb, 1992; Kornberg et al, 1992; Re et al, 1994; Ruoslahti and Reed, 1994; Schwartz, 1993). Normal endothelial and epithelial cells undergo apoptosis if cell attachment to substrate is blocked by culturing the cells in suspension or on surfaces unable to support anchorage and spreading (Frisch and Francis, 1994; Meredith et al, 1993; Re et al, 1994). These cells also become apoptotic if detached from their substrate using synthetic RGD peptides. Similarly, cultured fibroblasts incubated with antibody against the CD44 adhesion receptor detach from the substrate and undergo apoptosis (Henke et al, 1996).

In the present study, OPN-deficient VSMC did not undergo significant apoptosis when maintained in monolayer culture on plastic plates. However, a significant percentage of cells were apoptotic when there was no permanent adhesion to substrate. This occurred when cells were incubated in suspension for 24 hours or continuously exposed to collagen. In contrast, normal VSMC cultures or VSMC harboring the empty retroviral vector displayed minimal apoptosis when incubated under these conditions. This suggests that OPN-deficient VSMC are "relaxed" for apoptosis compared with normal VSMC. Continuous adhesion to plastic seems to be capable of suppressing the tendency toward apoptosis, whereas continuous exposure to collagen exacerbates it. The molecular basis for this enhanced predisposition to apoptosis remains to be determined.

Interestingly, cells exposed to collagen for 15 minutes and then incubated in suspension had lower rates of apoptosis than those incubated in suspension without any collagen exposure and those incubated for 15 minutes on plastic culture plates (6.9%, 37%, and 21%, respectively). In addition, cells exposed to collagen for only 15 minutes and subsequently incubated in suspension for 24 hours had approximately 50% in S-phase, whereas cells exposed for 30 minutes before incubation in suspension were either apoptotic or arrested in G_0. This raises the possibility that the intracellular signals generated by brief periods of collagen exposure may be protective and even growth-promoting, whereas longer periods of exposure are detrimental.

An intriguing feature of this study is the ability of exogenous OPN to reverse the adhesion defect on collagen gels. Complete rescue occurred only when exogenous OPN was added within 15 minutes of plating on collagen; no rescue was seen when OPN was added at 1 hour. This suggests that sufficient OPN must be present at or shortly after plating and that de novo OPN synthesis is unlikely to be important for mediating normal cell adhesion. In VSMC culture, OPN is not induced as a primary response gene and requires 6–12 hours of exposure to appropriate agonists (Giachelli et al, 1995; Green et al, 1995; Wang et al, 1996). In addition, treatment of normal VSMC with cycloheximide at or before trypsinization and replating on collagen gels did not affect adhesion, further suggesting that de novo synthesis of OPN is not normally necessary. Together with the time course for OPN rescue, it also argues that short-term effects of OPN on the synthesis of other proteins, such as integrins or matrix, are not likely to be involved either in the rescue of OPN-deficient clones or in the adhesion of normal VSMC to collagen.

Despite its ability to rescue adhesion, exogenous OPN conferred no protection against apoptosis. Addition of OPN to collagen gel cultures reduced the number of apoptotic cells by markedly increasing the percentage of adherent cells. However, the apoptotic fraction itself was not significantly altered for either adherent or nonadherent cells. Similarly, addition of OPN did not significantly alter the percentage of

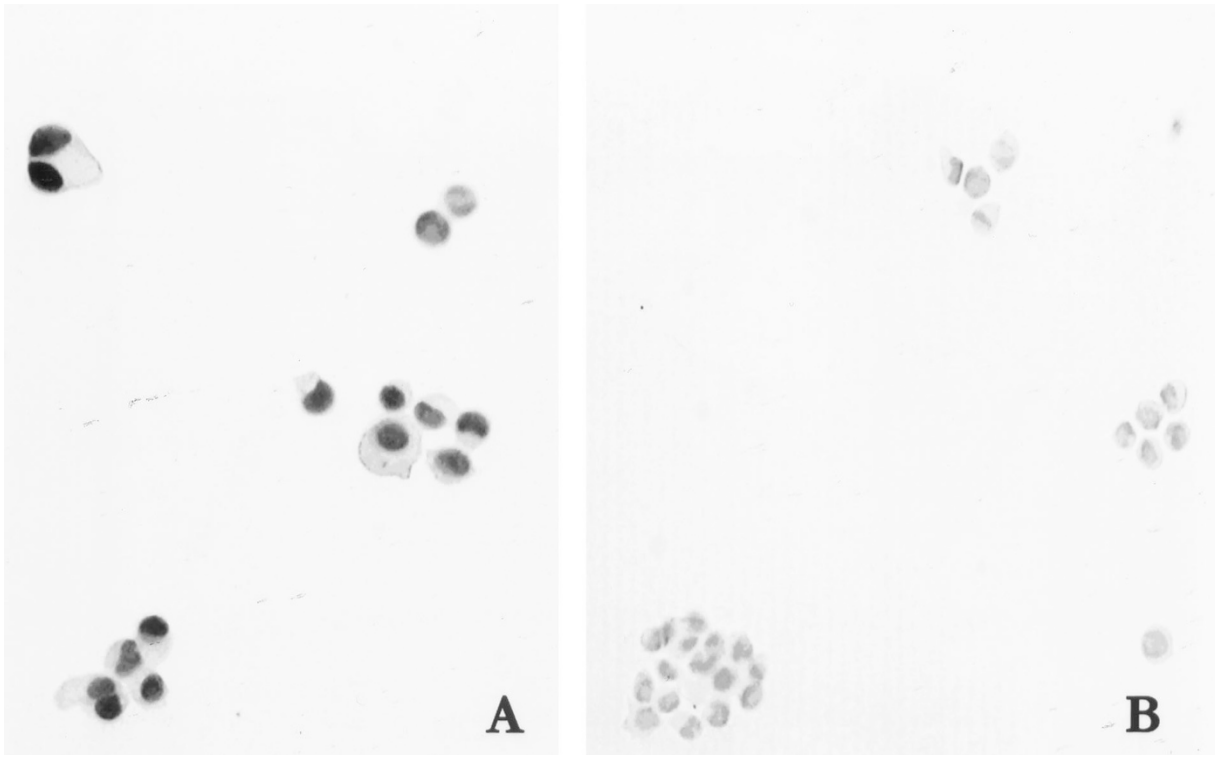

Figure 8.
In situ detection of apoptosis in collagen-exposed, nonadherent, OPN-deficient VSMC. Cells were stained using TUNEL with digoxigenin-conjugated dUTP. Nuclear DNA fragmentation was visualized using a peroxidase-substrate system and counterstained with methyl green. *A*, OPN-deficient VSMC after 1 hour of collagen exposure; *B*, OPN-deficient VSMC incubated in suspension without collagen exposure (Original magnification, ×40).

Table 4. Cell Cycle Distribution of OPN–Deficient VSMC Grown in Suspension for 24 Hours

	% APO	% G_0-G_1	% S	% G_2-M
OPN–deficient*	37.5	51	8.3	3
Normal VSMC*	0	80.7	3.4	15.6
Empty vector*	4.4	64.9	19.4	11.1
OPN–deficient† (plastic 15 minutes)	21.3	71	4.3	4.0
OPN–deficient + MC§	23.5	53	13.2	9.7
OPN–deficient + OPN 0_t‡	48.7	38.9	8.2	4.3

* OPN–deficient VSMC, uninfected VSMC (Normal) and VSMC infected with the empty retroviral vector without an OPN-antisense cDNA insert (Empty vector) were trypsinized, suspended in DMEM/10% CS without exposure to collagen, and incubated for 24 hours at 37° C in plastic conical tubes. Cells were then centrifuged, fixed, stained with PI, and analyzed for cell cycle distribution using flow cytometry.

† OPN–deficient VSMC were also trypsinized and replated onto plastic plates instead of collagen for 15 minutes. These cells were then released from the plates by EDTA and incubated in suspension for 24 hours at 37° C before cell cycle analysis.

§ OPN–deficient VSMC suspended in DMEM/10% CS without exposure to collagen were incubated for 24 hours at 37° C, in the presence of methylcellulose (MC).

‡ OPN–deficient VSMC suspended in DMEM/10% CS without exposure to collagen were incubated for 24 hours at 37° C in the presence of exogenous OPN (400 ng/ml).

apoptosis of OPN-deficient cells incubated in suspension without collagen exposure. This suggests that there are two distinct defects associated with OPN deficiency: an inability to adhere normally to collagen that seems to be mediated by $\alpha1\beta1$ and a baseline propensity for apoptosis that is suppressed by adhesion to plastic and aggravated by continuous exposure to collagen.

We propose the following model to explain the VSMC-collagen interactions described in this report (Fig. 9). Ligation of the full complement of $\alpha1\beta1$ receptors present on the surface of normal VSMC protects cells from apoptosis and mediates cell adhesion to collagen (Fig. 9A). The relative paucity of $\alpha1\beta1$ receptors on the surface of OPN-deficient VSMC does not allow most cells to permanently adhere to collagen. We hypothesize that during brief periods of collagen exposure, ligation of $\alpha1\beta1$ generates protective intracellular signals that inhibit apoptosis, even if the cells do not maintain anchorage (Fig. 9B). With longer collagen exposure in the absence of normal levels of $\alpha1\beta1$, ligation of other cell surface receptors (receptor X) may activate intracellular signals favoring cell death (Fig. 9C). Exogenous OPN may function as a "bridging" molecule between alternative integrin receptors (receptor Y) and OPN-deficient cells (Fig.

Table 5. Apoptosis in OPN–Deficient VSMC is Independent of Cell Density

Cell Density	% APO
1×10^6 cells/ml*	35.7
1×10^5 cells/ml	30
8.5×10^4 cells/ml	45
5×10^4 cells/ml	44.5
1×10^4 cells/ml	37

* Freshly trypsinized OPN–deficient VSMC were suspended in DMEM/10% CS at the cell densities indicated and incubated for 24 hours at 37° C in plastic conical tubes. Cells were then centrifuged, washed with PBS, fixed, stained with PI, and analyzed for cell cycle distribution using flow cytometry.

9D). The net effect is normal growth on collagen with low levels of apoptosis. An excess of exogenous OPN may also prevent activation of the apoptotic program by blocking interaction between collagen and receptor X. Equivalent doses of other RGD-containing proteins, such as fibronectin and vitronectin, are also able to rescue the adhesion defect on collagen gels, whereas chondroitin sulfate, which has no RGD, or laminin, which has a cryptic RGD site, did not (AS Weintraub and MB Taubman, unpublished observations). Thus, any protein with accessible RGD-sites may be able to facilitate binding to receptor Y.

Increasing evidence supports an important role for apoptosis in arterial remodeling during normal angiogenesis and in atherosclerosis (Bennett et al, 1995; Cho et al, 1995; Geng and Libby, 1995; Han et al, 1995; Isner et al, 1995). VSMC apoptosis may limit the extent of the lesion or accelerate regression of lesion size (Bochaton-Piallat et al, 1995; Han et al, 1995). On the other hand, apoptosis of plaque VSMC may render the fragile fibrous cap of the plaque more vulnerable to rupture, by removing the synthetic source of ECM components that give the plaque stability (Bennett et al, 1995; Geng and Libby, 1995). OPN may play an important role in allowing VSMC to interact normally with collagen and in protecting them from apoptosis. It remains to be determined whether OPN plays either role in vivo and whether this is beneficial or detrimental.

Materials and Methods

Reagents

Purified hamster anti-mouse α5 (HMa5–1), β1 (HMb1–1), and purified mouse anti-rat β3 (F11) mAbs were purchased form PharMingen (San Diego, California). Purified hamster anti-rat α1 (HA 3/18) and α2 (HA 1/29) mAb and isotype-specific control IgG (HA 4/8) were a generous gift of Dr. Phil Gotwals and Dr. Victor Koteliansky (Biogen, Cambridge, Massachusetts). Purified rat OPN protein was provided by Dr. Cecilia Giachelli (University of Washington, Seattle, Washington). Methylcellulose and cycloheximide were purchased from Fluka BioChemika (Milwaukee, Wisconsin) and Sigma (St. Louis, Missouri), respectively.

Cell Culture

VSMC were isolated from the thoracic aortas of 200–300 gram male Sprague-Dawley rats by enzymatic dissociation as previously described (Taubman et al, 1993). Rat aortic VSMC were infected with the pMV12 retroviral expression vector (Cacace et al, 1993) containing the full length OPN cDNA in the antisense orientation. Four infectants (AS 2/7, AS-1 2/22, AS 2/22, and AS 2/28) stably underexpressing OPN were described previously (Weintraub et al, 1996) and used for experiments described in this report. Normal VSMC and/or VSMC infected with the pMV12 vector lacking an OPN cDNA insert were used as controls. For maintenance, VSMC lines were grown at 37° C in 5% CO_2 on 100 mm plates containing DMEM supplemented with 10% heat-inactivated CS, 100 U/ml penicillin, and 100 mg/ml streptomycin, and serially passaged before reaching confluence.

In some experiments, normal or OPN-deficient VSMC were incubated in suspension. For these studies, monolayers were trypsinized with 0.05% trypsin-EDTA, centrifuged at 600 $\times g$ for 5 minutes at 4° C, washed with 5 ml of PBS, and recentrifuged for 5 minutes. Cell pellets were resuspended in 10 ml of DMEM/10% CS and incubated in 50 ml plastic conical tubes with loose fitting caps at 37° C in 5% CO_2.

Cell Counting

Cells isolated by trypsinization and centrifugation were resuspended in 1 ml of DMEM/10% CS. Aliquots (10 μl) were counted in quadruplicate with a hemacytometer (Fisher Scientific, Pittsburgh, Pennsylvania). Cells incubated in suspension were centrifuged, washed with 5 ml of PBS, and re-centrifuged before counting.

Extracellular Matrices

Three dimensional collagen matrices were constructed in 12-well dishes or 100 mm plates using a modification of previously described methods (Boudreau et al, 1991; Wren et al, 1986). Vitrogen 100 (Celtrix, Santa Clara, California), a solution of nondenatured, pepsin-solubilized bovine dermal collagen with an intact triple-helical structure, was combined with 1× and 10× M199 medium (GIBCO, Grand Island, New York) in 3.5:1 ratio (Boudreau et al, 1991), for a final collagen concentration of 2 mg/ml, and adjusted to pH 7.4. Gels polymerized overnight at 37° C and were primed with DMEM/10% CS before use. Matrigel-coated tissue culture plates (Biocoat Cellware, Becton Dickinson Labware, Bedford, Massachusetts) were rehydrated with warm DMEM according to the manufacturer's instructions before use. Before plating onto collagen gels, VSMC were trypsinized, centrifuged, and counted. VSMC were released from collagen gels by digestion with Type II collagenase (Worthington Biochemical Corporation, Lakewood, New Jersey) at 37° C for 1 hour.

Table 6. Effect of Exogenous OPN on Apoptosis of OPN–Deficient VSMC During Continuous Collagen Exposure

	% Apoptosis			
	% Adherent‡	Adherent cells§	Nonadherent cells†	Total Apo
No OPN*	10	10.7	92.5	84.3
OPN at 0_t≠	90	10.2	77	16.9
OPN at 15 minutes≠	86	ND	78.5	–

* OPN–deficient VSMC were trypsinized, suspended in DMEM/10% CS, and incubated on collagen gels for 24 hours at 37° C.

‡ The culture medium was collected 24 hours after plating and cell number was determined. % Adherent = (number of cells plated − number of cells in culture medium) ÷ number of cells plated.

† After incubation, nonadherent cells in the culture medium were fixed, stained with PI, and analyzed for cell cycle distribution using flow cytometry.

§ After incubation, the collagen gels were digested with Type II collagenase to release the adherent cells. These cells were also fixed, stained with PI, and analyzed for cell cycle distribution.

≠ Exogenous OPN (400 ng/ml) was added to the cultures at the time of plating (0_t) or 15 minutes after plating.

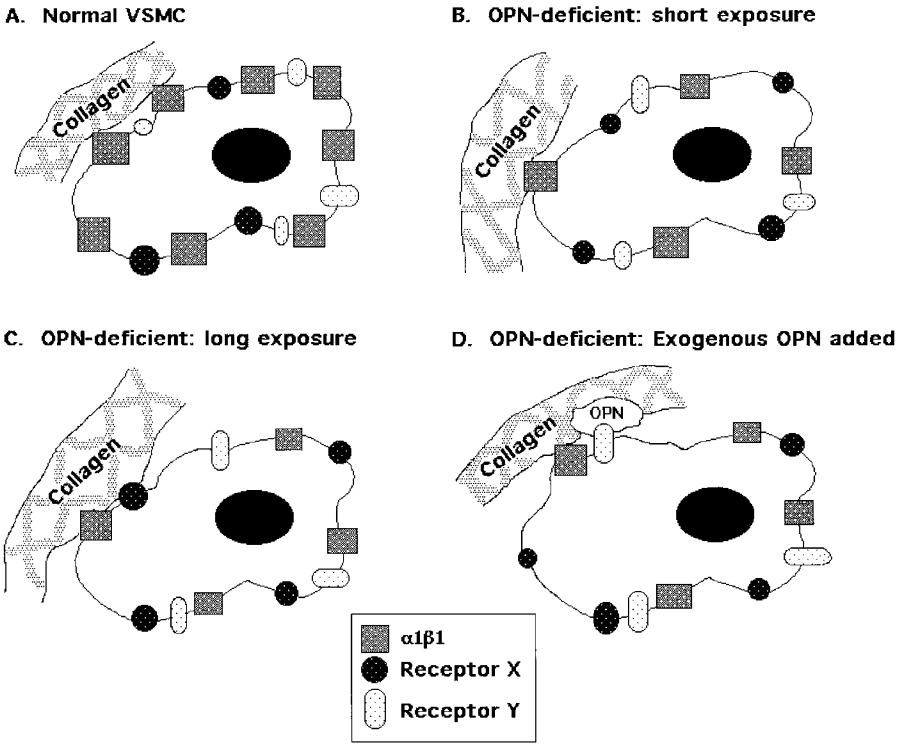

Figure 9.

Potential mechanisms of OPN-collagen interactions. *A*, Ligation of the full complement of $\alpha1\beta1$ receptors on the surface of normal VSMC protects cells from apoptosis and mediates cell adhesion. *B*, Short exposure of OPN-deficient VSMC to collagen allows ligation of $\alpha1\beta1$ receptors, which inhibits apoptosis. The paucity of receptors, however, does not permit permanent adhesion. *C*, Longer exposure of OPN-deficient cells to collagen in the absence of normal levels of $\alpha1\beta1$ results in ligation of Receptor X and $\alpha1\beta1$. This may generate net intracellular signals favoring programmed cell death. *D*, Exogenous OPN may function as a "bridging" molecule between alternative integrin receptors and OPN-deficient cells to maintain adhesion and allow normal growth on collagen without apoptosis.

Cell Adhesion

VSMC were plated onto collagen matrices in DMEM/10% CS. The culture medium was removed at various time points after plating, and the gels were washed vigorously with phosphate buffered saline (PBS). Cell counts were performed in quadruplicate on the combined medium/washes. % Adherent = (number of cells plated − cells in culture medium) ÷ number of cells plated.

Cell Cycle Analysis

At varying time points, cells were fixed in cold 70% ethanol, rinsed with PBS, and resuspended in a fresh PI (10 μg/ml; Sigma)-RNAse A (0.5 mg/ml; Sigma) staining solution. Samples were analyzed for DNA content and cell cycle profile using a FACScan flow cytometer (Becton Dickinson), with red fluorescence excitation at 488 nm wavelength. Data analysis was performed using LYSIS II and ModFit software (Verity, Maine). Each experiment was performed at least three times.

In Situ Detection of Apoptosis

Cells were fixed in cold methanol/acetone (1:1) and air-dried onto Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, Pennsylvania). Nuclear

DNA fragmentation was detected using a TUNEL assay, with digoxigenin-conjugated dUTP (ApopTag, Oncor, Gaithersburg, Maryland), according to the manufacturer's instructions.

Cell Surface Integrin Expression

OPN-deficient cells grown to confluence on plastic culture plates were isolated by trypsinization and centrifugation. Cell pellets were washed with PBS, resuspended in normal goat serum, and then incubated with mAbs directed against α1 (3.66 mg/ml), α2 (3.99 mg/ml), α5 (0.5 mg/ml), β1 (0.5 mg/ml), or β3 (0.5 mg/ml) integrin subunits, or with isotype-specific nonimmune IgG (3 mg/ml). After three PBS washes, cells were incubated with a fluorescein-isothiocyanate (FITC) conjugated goat anti-hamster IgG (Southern Biotechnology Associates, Birmingham, Alabama). As controls, normal VSMC and VSMC infected with empty pMV12 vector were labeled as described above. Background cell autofluorescence was assessed by omitting the primary antibody. MFI were determined at 488 nm using EPICS Profile II flow cytometer with Elite software (Coulter Electronics, Hialeah, Florida) and normalized for nonspecific antibody binding.

RNA Blot Hybridization

Isolation of total RNA from VSMC, agarose gel electrophoresis, transfer to nitrocellulose, and hybridization to ^{32}P-labeled cDNA were performed as previously described (Wax et al, 1994). Final washes were in 0.5 $\times$ SSC and 0.1% SDS at 42° C for 15 minutes. The α1 cDNA was labeled with [^{32}P]dCTP (800 Ci/mmol, Amersham Corporation, Arlington Heights, Illinois) by random oligomer priming to a specific activity of greater than 10^8 cpm/mg and was hybridized at 2–3 $\times$ 10^6 cpm/ml. Equal loading of lanes was verified by staining the 18S and 28S ribosomal RNA with ethidium bromide.

Statistics

Values for MFI are expressed as mean $\pm$ standard deviation. Data were analyzed by one-way analysis of variance to test for differences in MFI involving multiple clones. Differences between individual conditions were assessed using a post-hoc analysis with Fisher's Protected Least Significant Difference (PLSD). StatView 4.01 software (Abacus Concepts, Berkeley, California) was used for all statistical analyses

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