Inhibition of Vascular Smooth Muscle Cell Growth by Inhibition of Fibronectin Matrix Assembly

Kwesi O. Mercurius, Alex O. Morla

Abstract—The regulation of vascular smooth muscle cell (VSMC) proliferation by the fibronectin matrix was tested by treating human umbilical artery smooth muscle cells (HUASMCs) with a recombinant fragment of fibronectin (protein III1-C) that has previously been shown to modulate fibronectin matrix assembly. III1-C inhibited HUASMC proliferation by 75% to 90%. The inhibition of growth was time dependent; III1-C had no effect on DNA synthesis after 0 to 5 hours of treatment but did have an effect at 24 hours and beyond. III1-C did not stimulate apoptosis in these cells, indicating that the inhibition of proliferation was not due to an induction of programmed cell death. The effects of III1-C on cell growth were only specific for normal diploid smooth muscle cells. III1-C had no effect on the proliferation of IMR-90 fibroblasts, endothelial cells, NIH 3T3 cells, or the rat aortic smooth muscle cell line A7r5. However, III1-C did inhibit proliferation by primary rat aortic smooth muscle cells. An analysis of HUASMC fibronectin receptor (integrin α5β1) distribution revealed that III1-C did not inhibit α5β1 localization to focal contacts. Moreover, III1-C had no effect on the relative expression levels of seven different integrin subunits on HUASMCs. However, III1-C did inhibit fibronectin matrix assembly by rat aortic smooth muscle cells, HUASMCs, A7r5 cells, IMR-90 cells, and endothelial cells. An analysis of fibronectin synthesis indicated that the inhibition of fibronectin matrix assembly by III1-C was not due solely to a decrease in fibronectin synthesis. Finally, treatment of HUASMCs with anti-fibronectin monoclonal antibody L8 (which is known to inhibit fibronectin matrix assembly) also decreased the rate of HUASMC DNA synthesis. These results demonstrate that III1-C inhibits VSMC proliferation and suggest that this effect may be mediated by the inhibition of fibronectin matrix assembly. (Circ Res. 1998;82:548-556.)

Key Words: extracellular matrix • integrin • laminin • Matrigel

The ECM is known to have a great influence on the growth and differentiation of various cell types. For example, the ECM is required for the survival of endothelial and epithelial cells and for the proliferation of fibroblasts. Moreover, the expression of differentiation-specific proteins (eg, β-casein) by mammary epithelial cells requires a specific combination of growth factors and hormones plus the right ECM substratum.

The ECM has also been found to regulate VSMC phenotype. VSMCs that are removed from their tissue of origin and placed in cell culture gradually modulate from a contractile to a synthetic phenotype in a manner that is thought to recapitulate the modulation seen in disease states in vivo. ECM molecules such as collagen, FN, and laminin can influence the phenotypic modulation of VSMCs in culture. For example, VSMCs grown on fibrillar collagen matrices do not proliferate, whereas those grown on monomeric collagen coated onto a dish do proliferate. This inhibition of VSMC proliferation by fibrillar collagen has been found to be due to upregulation of cdk2 inhibitors.

Another example of the regulation of VSMC modulation by ECM molecules can be seen with laminin and FN. VSMCs that are cultured on laminin retain a contractile phenotype longer than do cells cultured on FN. Moreover, cells cultured on laminin plus synthetic peptides containing the RGD integrin—binding motif found in FN retain a contractile phenotype longer than do cells cultured on laminin alone. The experiments described above do not distinguish whether the maintenance of a contractile phenotype was due to the inhibition of integrins binding to FN or to the inhibition of FN matrix assembly.

FN matrix assembly is a cell-mediated process that involves the binding of FN to cell surface receptors (integrins) and to cell-associated FN molecules. The interaction between the FN receptor integrin α5β1 and the RGD site of FN is required for matrix assembly in most cellular systems. However, other RGD-binding integrins (eg, αIIbβ3 and αvβ3) can support FN matrix assembly.

FN matrix assembly also requires specific FN-FN binding interactions at the cell surface. The regions in FN that have been shown to be involved in FN-FN binding and are required for proper matrix assembly of the molecule include the N-terminal 70-kD region, the first type III repeat, and the 10th type III repeat. We have previously identified a recombinant protein representing a C-terminal portion of the
first type III repeat in FN (protein III₁₋C) that is able to induce spontaneous in vitro disulfide cross-linking of FN. The III₁₋C protein also enhances FN matrix assembly on CHO cells and enhances the binding of cells to FN.

As described above, RGD peptides enhance the maintenance of the contractile phenotype in VSMCs cultured on laminin; however, it is not known whether this is due to the inhibition of integrins binding to FN or to the inhibition of FN matrix assembly. Moreover, although culturing cells on dimeric FN is known to accelerate VSMC phenotypic modulation, the influence of VSMC FN matrix assembly on growth and modulation is not known. In the present study, we have used the III₁₋C protein to alter the FN matrix assembly of VSMCs to test the role of FN matrix assembly on VSMC growth. The results indicate that III₁₋C inhibits VSMC FN matrix assembly and that this leads to an inhibition of cell proliferation.

Materials and Methods

Materials

HUASMCs and Sm-GM2 medium were purchased from Clonetics. IMR-90 human lung fibroblasts (ATCC CCL-186), NIH 3T3 fibroblasts (ATCC CRL-1685), and A7r5 rat aortic SMCs (ATCC CRL-1444) were obtained from the American Type Culture Collection. DMEM, glutamine Pen-Strep, and mouse laminin were acquired from Life Technologies, Inc. FBS was purchased from Sigma Chemical. Protein A Sepharose was from Pharmacia LKB. All other reagents were purchased from Sigma.

Cell Culture

HUASMCs were cultured in Sm-GM2 medium, which contains 5% FCS, 10 ng/mL human epidermal growth factor, 2 ng/mL human fibroblast growth factor, 5 µg/mL insulin, 50 µg/mL gentamicin, and 50 ng/mL amphotericin. HUASMCs were typically between passages 5 and 9 for experiments. Primary rat aortic SMCs were a kind gift of Dr Michael Parmacek (University of Chicago, Ill) and were grown in either Sm-GM2 or 50% DMEM, 50% Ham's F-12 plus 10% FBS, and glatimine Pen-Strep. HUVECs were isolated from umbilical veins as described previously. HUVECs were cultured in Dulbecco's medium supplemented with 20% FBS, 50 µg/mL heparin, 30 µg/mL endothelial cell growth supplement, and glutamine Pen-Strep and were in passage 1 for the present study. IMR-90 cells, NIH 3T3 cells, and A7r5 cells were cultured in DMEM supplemented with 10% FBS and glutamine Pen-Strep. IMR-90 cells were in passage 12 for the present study.

Recombinant Protein Production

Proteins III₁₋C and III₁₁₋C (previously called III₁₁, Ref 37) were produced in bacteria and purified as described previously. Stock protein solutions were typically 800 µmol/L for III₁₋C and 260 µmol/L for III₁₁₋C in PBS.

Cell Growth Analyses

For DNA synthesis analysis, wells of 24-well cluster dishes (Fisher) were coated with 20 µg/mL of rabbit polyclonal anti-human FN IgG in 5% goat serum/Tris-buffered saline (TBS). Cells were cultured as described for DNA synthesis analysis above, except that glass coverslips were placed in the wells of the 24-well dishes before coating with ECM proteins. After 2 to 4 days in culture, cells were fixed for 30 minutes at 37°C. III₁₋C, III₁₁₋C, or rabbit anti-human FN antibodies were added to the appropriate wells, and the cells were cultured in the presence of these treatments for the duration of the experiment. At the appropriate time, the rate of DNA synthesis was measured by 1-hour pulse labelings with 10 µCi/mL [3H]thymidine as previously described. Student's t test was performed to determine the statistical significance of the differences seen between III₁₋C–treated cultures and control-treated cultures.

The effect of III₁₋C on cell growth was also measured by determining the increase in cell number of the cultures. Wells of six-well cluster dishes either were left uncoated or were coated with 20 µg/mL FN, 20 µg/mL laminin, or 100 µg/mL growth factor–reduced Matrigel. All protein solutions were in PBS. After coating at RT for 1 hour, cells were washed extensively with PBS, and then cells were added. Cells were plated at a density of 1.5 to 3x10⁵ per well and then allowed to attach and spread for 30 minutes at 37°C. III₁₋C, III₁₁₋C, or mAb L8 was then added to the appropriate wells, and the cells were cultured in the presence of these treatments for the duration of the experiment. At the appropriate time, the rate of DNA synthesis was measured by 1- or 2-hour pulse labelings with 10 µCi/mL [3H]thymidine as previously described. Student's t test was performed to determine the statistical significance of the differences seen between III₁₋C–treated cultures and control-treated cultures.

Immunofluorescence Assays and Quantification of FN Matrix Assembly

Cells were cultured as described for DNA synthesis analysis above, except that glass coverslips were placed in the wells of the 24-well dishes before coating with ECM proteins. After 2 to 4 days in culture, cells were fixed for 30 minutes at RT with 3.7% paraformaldehyde and 60 mmol/L sucrose in PBS. Cells were then washed with 0.5% Triton X-100 in TBS. After coating at RT for 1 hour, cells were washed extensively with PBS, and then cells were added. Cells were plated at a density of 1.5 to 3x10⁵ per well and then allowed to attach and spread for 30 minutes at 37°C. III₁₋C or III₁₁₋C was then added to the appropriate wells, and the cells were cultured in the presence or absence of these recombinant proteins for the duration of the experiment. After 1, 3, and 7 days of culturing, cells were harvested by trypsinization, and the number of cells in each population was measured with a hemocytometer.

Materials and Methods

Selected Abbreviations and Acronyms

CHO = Chinese hamster ovary
DAPI = 4',6-diamidino-2-phenylindole
ECM = extracellular matrix
FACS = fluorescence-activated cell sorter
FN = fibronectin
HUASMC = human umbilical artery SMC
HUVEC = human umbilical vein endothelial cell
RT = room temperature
SMC = smooth muscle cell
TBS = Tris-buffered saline
TCA = trichloroacetic acid
TUNEL = terminal deoxynucleotidyl transferase–mediated dUTP-digoxigenin nick end labeling
VSMC = vascular SMC

Protein A Sepharose was from Pharmacia LKB. All other reagents were purchased from Sigma.

Cell Culture

HUASMCs were cultured in Sm-GM2 medium, which contains 5% FCS, 10 ng/mL human epidermal growth factor, 2 ng/mL human fibroblast growth factor, 5 µg/mL insulin, 50 µg/mL gentamicin, and 50 ng/mL amphotericin. HUASMCs were typically between passages 5 and 9 for experiments. Primary rat aortic SMCs were a kind gift of Dr Michael Parmacek (University of Chicago, Ill) and were grown in either Sm-GM2 or 50% DMEM, 50% Ham's F-12 plus 10% FBS, and glutamine Pen-Strep. HUVECs were isolated from umbilical veins as described previously. HUVECs were cultured in Dulbecco’s medium supplemented with 20% FBS, 50 µg/mL heparin, 30 µg/mL endothelial cell growth supplement, and glutamine Pen-Strep. HUVECs were in passage 1 for the present study. IMR-90 cells, NIH 3T3 cells, and A7r5 cells were cultured in DMEM supplemented with 10% FBS and glutamine Pen-Strep. IMR-90 cells were in passage 12 for the present study.

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The effect of III₁₋C on cell growth was also measured by determining the increase in cell number of the cultures. Wells of six-well cluster dishes either were left uncoated or were coated with 20 µg/mL FN as described above. HUASMCs were plated at a density of 10 000 cells per well and then allowed to attach and spread for 30 minutes at 37°C. III₁₋C or III₁₁₋C was then added to the appropriate wells, and the cells were cultured in the presence or absence of these recombinant proteins for the duration of the experiment. After 1, 3, and 7 days of culturing, cells were harvested by trypsinization, and the number of cells in each population was measured with a hemocytometer.

Immunofluorescence Assays and Quantification of FN Matrix Assembly

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AFCScan flow cytometer (Becton Dickinson). The data were also analyzed by Student’s t test.

**Apoptosis Assays**

Cells were cultured on coverslips as described for immunofluorescence analysis above. At the appropriate times, cells were fixed for 30 minutes at RT with 3.7% parafomaldehyde and 60 mmol/L sucrose in TBS. Cells were washed three times with PBS, permeabilized with 0.5% Triton X-100 and 0.1% sodium citrate in PBS, washed again, and then incubated for 1 hour at 37°C with the TUNEL assay solution (from the In Situ Cell Death Detection Kit, Fluorescein, Boehringer-Mannheim). Cells were then washed two times with PBS and incubated for 20 seconds with 1 mmol/L DAPI in PBS. Cells were washed two times with PBS, and the coverslips were mounted and analyzed with a ×20 oil-immersion objective on a Zeiss Axioskop fluorescence microscope.

**FN Synthesis Assay**

HUASMCs were cultured as described above for DNA synthesis assays, except that 10-cm-diameter dishes were used instead of 24-well plates. After 24 hours of culturing in the presence or absence of either III1-C or III11-C, cells were washed two times, 5 minutes per wash, with Met/Cys-free DMEM+10% FBS+glutamine Pen-Strp. Cells were then cultured in 3 mL of fresh Met/Cys-free medium±III1-C or III11-C (where appropriate) plus 200 μg/mL 5-bromo-2′-deoxyuridine for 30 minutes at 37°C. Plates were washed with ice-cold PBS and then lysed in RIPA buffer plus protease inhibitor cocktail. Lysates were precleared by centrifugation at 12,000g for 15 minutes at 4°C, and then the amount of 35S incorporated into TCA-precipitable counts was measured. Lysates were brought to equal TCA-precipitable counts per milliliter, and then FN was immunoprecipitated with polyclonal anti-FN IgG. The antibodies were collected onto protein A Sepharose beads, washed four times with RIPA, and then boiled in SDS-PAGE sample buffer containing 2-mercaptoethanol. Samples were electrophoresed on a 4% to 20% polyacrylamide gradient gel, the gel was dried, and the radioactive proteins were detected by an 18-hour exposure with a Molecular Dynamics PhosphorImager.

**Integrin Expression Analysis**

HUASMCs were cultured as described above for DNA synthesis assays, except that cells were cultured on 10-cm-diameter dishes instead of 24-well dishes (cells were seeded at 3×10⁵ cells per 10-cm dish). After 24 hours of culturing in the presence or absence of either III1-C or III11-C, cells were harvested by trypsinization and then counted. Each point represents the mean of triplicate samples. Statistical analysis was performed by using Student’s t test. *P<.05 and **P≤.01 vs control.

**Results**

The recombinant FN fragment III1-C was used to test the role of FN matrix assembly on VSMC growth. HUASMCs were cultured on FN-coated dishes in either the presence or absence of III1-C. As shown previously, 13–15 VSMCs cultured on FN-coated dishes in either the presence or absence of III1-C proliferated at a higher rate than do cells cultured on uncoated dishes (Fig 1A, bars labeled FN and III1-C). After 3 days, the rate of DNA synthesis was measured by a 2-hour pulse labeling with [3H]thymidine, as described in “Materials and Methods.” The data represent the mean and standard deviation of triplicate cultures for each condition. B, HUASMCs were cultured in uncoated wells (control, □) or in FN-coated wells plus either no added protein (FN, △), 50 μmol/L III1-C (FN+11C), or 50 μmol/L III11-C (FN+11C, ◆). After 1, 3, and 7 days, cells were trypsinized and counted. Each data point represents the mean of triplicate samples. Statistical analysis was performed by using Student’s t test. *P<.05 and **P≤.01 vs control.

The effect of III1-C on total cell number was consistent with the effect of the peptide on DNA synthesis shown above. Over
that III1-C inhibits HUASMC growth at a specific stage in the cell cycle (eg, at the G1- to S-phase transition). Thus, if III1-C disrupts the adhesion to the ECM, this could potentially lead to cell cycle arrest. The main FN receptor on SMCs is the integrin α5β1; thus, we analyzed the effect of III1-C on α5β1-FN interactions. The only ECM ligand for α5β1 is FN, and when α5β1 is bound to FN, the integrin becomes concentrated in focal adhesions.

To determine whether III1-C could inhibit the growth of other cell types, we tested the effect of the peptide on primary rat aortic SMCs, the A7r5 rat VSMC line, IMR-90 fibroblasts, and HUVECs. Treatment of IMR-90 fibroblasts and HUVECs with III1-C resulted in no change in DNA synthesis (not shown). Therefore, one measure of the inhibition of DNA synthesis must be due to a mechanism other than induction of programmed cell death.

To test this, HUASMCs were treated with or without III1-C for 1 to 4 days, and at each day the percentage of cells in apoptosis was determined by DAPI staining of DNA and by TUNEL assay. All cultures gave similar results: <1% of the cells were apoptotic in all of the culture conditions (not shown). Thus, III1-C does not induce HUASMC apoptosis, and the inhibition of DNA synthesis must be due to a mechanism other than induction of programmed cell death.

To determine whether III1-C could inhibit the growth of other cell types, we tested the effect of the peptide on primary rat aortic SMCs, the A7r5 VSMC line, IMR-90 and NIH 3T3 fibroblasts, and endothelial cells. Treatment of IMR-90 fibroblasts and HUVECs with III1-C resulted in no change in DNA synthesis compared with untreated or control-treated cultures (Fig 3). III1-C also did not inhibit the growth of NIH 3T3 cells (not shown). In addition, III1-C had no significant effect on the proliferation of an established rat VSMC line, A7r5 (Fig 3). However, III1-C did significantly inhibit the proliferation of primary rat aortic SMCs (Fig 3). These results indicate that III1-C does not inhibit the growth of various cell cultures and cell lines but does inhibit the growth of normal diploid rat and human VSMCs.

The mechanism by which III1-C inhibits VSMC growth was addressed by analyzing the effect of III1-C on FN receptors. For nontransformed fibroblasts it has been shown that cell adhesion to the ECM is essential for progression through the cell cycle.40,41 Thus, if III1-C disrupts the adhesion of VSMCs to the FN-coated on the dish, this could potentially lead to cell cycle arrest. The main FN receptor on SMCs is the integrin α5β1; thus, we analyzed the effect of III1-C on α5β1-FN interactions. The only ECM ligand for α5β1 is FN, and when α5β1 is bound to FN, the integrin becomes concentrated in focal adhesions.42–45 Moreover, when α5β1 is not bound to FN, it remains diffusely distributed across the cell membrane, even if the cell contains focal contacts.46 Thus, the only time α5β1 is found in focal contacts is when it is bound to its ligand, FN, in the ECM. Therefore, one measure of α5β1 binding to FN in cultured cells is to determine the subcellular distribution of α5β1; localization of α5β1 to focal contacts indicates that this integrin is bound to FN. Anti-phosphotyrosine antibodies were used to identify the locations of focal contacts, as has been previously shown.46–48 In control

Figure 2. Time course of effect of III 1-C on HUASMC DNA synthesis. HUASMCs were cultured in uncoated wells (control, □) or in FN-coated wells plus either no added protein (FN, ◀), 50 μmol/L III1-C (FN+C, ◆), or 50 μmol/L III11-C (FN+11C, ◇). At 0, 3, 5, 24, and 48 hours after adding III1-C to the cultures, the rate of DNA synthesis was measured by a 1-hour pulse labeling with [3H]thymidine, as described in “Materials and Methods.” At each time point, the rate of [3H]thymidine incorporation for each sample was compared with the rate for the control culture, which was set to 100%. Each data point represents the mean of triplicate samples. Statistical analysis was performed by using Student’s t test. *P≤.05 vs control.

Figure 3. Effect of III1-C on the DNA synthesis of various cell types. The effect of III1-C on the rate of DNA synthesis of HUASMCs, rat aortic SMCs (RASMCs), the A7r5 rat aortic SMC line, IMR-90 fibroblasts, and HUVECs was determined as described in the legend to Fig 1. For this experiment, cells were cultured in the absence or presence of III1-C or III11-C for 4 days before measuring [3H]thymidine incorporation. The data represent the mean and standard deviation of triplicate cultures for each condition. Statistical analysis was performed by using Student’s t test. **P≤.01 vs control.
cells seeded onto uncoated plastic, α5β1 was diffusely distributed, even though these cells did contain prominent focal contacts (Fig 4, control panels). A few focal contacts did contain α5β1, but most focal contacts in the control cultures were devoid of α5β1. This is consistent with the well-established finding that cells seeded onto uncoated culture dishes in the presence of serum attach to and spread on vitronectin from the serum that has adhered to the culture dish and that the main vitronectin receptors in VSMCs are αv-containing integrins (not α5β1).

In contrast to the results described above with uncoated wells, most of the focal adhesions in cells cultured on FN-coated wells contained α5β1 (Fig 4, FN, FN+C, and FN+11C panels). Note that in the cultures treated with III1-C, the α5β1 staining of the focal contacts was perhaps less intense than in the cultures treated with the control protein III11-C. However, most of the focal contacts in these cells did contain α5β1. This result indicates that III1-C does not inhibit the localization of α5β1 to focal contacts.

The effect of III1-C on overall integrin expression was also determined. III1-C- and III11-C–treated HUASMCs were subjected to FACS analysis with a battery of anti-integrin antibodies. HUASMCs were found to express α1β1, α2β1, α3β1, α5β1, and αvβ3, but no αvβ5 (Fig 5 shows results with anti-α2, -α3, -α5, and -β1) (note that the cells may also express αvβ1, but the available antibodies cannot specifically distinguish that integrin subunit combination in a FACS analysis). Treatment of HUASMCs with III1-C resulted in no significant change in integrin expression (Fig 5; α1, αv, and β3 also did not change but are not shown). Thus, III1-C did not reduce relative integrin expression levels on the VSMCs.

The mechanism by which III1-C inhibits VSMC growth was addressed next by analyzing the effect of III1-C on FN matrix assembly. All of the cell types tested (HUASMCs, A7r5 cells, IMR-90 cells, and HUVECs) assembled an FN matrix, although the most extensive fibrils were produced by the HUASMCs and the IMR-90 fibroblasts (Fig 6, control panels). Cells grown on FN or additionally treated with the control protein III11-C also assembled fibrillar FN matrices (Fig 6, FN and FN+11C panels). By contrast, III1-C–treated cells in each case produced short, faint, fibril-like stitches that stained with anti-FN antibodies but had none of the typical prominent FN fibrils seen in the control cultures (Fig 6, FN+C panels).

The effect of III1-C on FN matrix assembly was quantified next. Rat aortic SMCs were grown in the presence of either III1-C or III11-C for 3 days, and then the cultures were analyzed by immunofluorescence with anti-FN antibodies as described above. The amount of area occupied by FN fibrils was quantified in randomly chosen fields. The data demonstrate that III1-C inhibited FN matrix assembly by ~90% (Fig 7). The results shown in Figs 6 and 7 indicate that III1-C significantly inhibited FN fibril formation in each of the cell types tested.

One way in which III1-C could potentially reduce FN matrix assembly is by inhibiting FN synthesis. The effect of III1-C on HUASMC FN synthesis was studied by labeling proteins with 35S-translabel and then immunoprecipitating with anti-FN antibodies. The 35S labeling revealed that overall protein synthesis was 60% to 65% lower (on a per-cell basis) in...
in III1-C–treated cells than in control-treated cells (data not shown). However, III 1-C had no specific effect on FN synthesis. When cell lysates were equalized for total TCA-precipitable counts, the amount of 35S-labeled FN produced from III 1-C–treated and III 11-C–treated cells was equivalent (Fig 8). Therefore, although III 1-C has no specific effect on the synthesis of FN, III 1-C treatment does result in an overall reduction in protein synthesis. The net effect is that III1-C–treated cells produce 60% to 65% less FN per cell than do control-treated cultures. Although this may play a part in the inhibited FN matrix assembly seen in III1-C–treated cells, it may not account for the entire inhibition of matrix assembly, because FN synthesis is only partially inhibited and because cells are able to produce FN fibrils from the FN found in the medium and the FN coated on the dish.17 In any case, the above data indicate that III 1-C inhibited FN matrix assembly and decreased overall protein synthesis but did not have any specific effect on FN synthesis. Thus, the inhibition of FN matrix assembly may be due in part to the decrease in overall protein synthesis and in part to competition during fibrillogenesis by direct interactions between III 1-C and FN.32,35,36

The matrix assembly results shown in Figs 6 and 7 suggest that III 1-C may inhibit VSMC proliferation by inhibiting FN matrix assembly. If that is the case, other methods of inhibiting FN matrix assembly might also be expected to inhibit the proliferation of these cells. To test this hypothesis, HUASMCs were treated with the anti-FN mAb L8, which has previously been shown to inhibit FN matrix assembly.49 Treatment of HUASMCs with mAb L8 resulted in a concentration-dependent inhibition of DNA synthesis (Fig 9), indicating that inhibition of FN matrix assembly leads to inhibition of HUASMC proliferation.

Discussion

The results presented above demonstrate that III 1-C inhibits VSMC growth. This effect was cell-type specific, since normal diploid HUASMCs and rat aortic SMCs were affected, but IMR-90 cells, A7r5 cells, NIH 3T3 cells, and HUVECs were not affected. III 1-C did not reduce the relative expression of FN receptors on VSMCs. Although this may play a part in the inhibited FN matrix assembly seen in III1-C–treated cells, it may not account for the entire inhibition of matrix assembly, because FN synthesis is only partially inhibited and because cells are able to produce FN fibrils from the FN found in the medium and the FN coated on the dish.17 In any case, the above data indicate that III 1-C inhibited FN matrix assembly and decreased overall protein synthesis but did not have any specific effect on FN synthesis. Thus, the inhibition of FN matrix assembly may be due in part to the decrease in overall protein synthesis and in part to competition during fibrillogenesis by direct interactions between III 1-C and FN.32,35,36

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Rat aortic SMCs were cultured in the presence of C or III1-C (11c) as described in the legend to Fig 6. After 3 days in culture, the cells were fixed and processed for indirect immunofluorescence with polyclonal anti-FN antibodies as described in “Materials and Methods.” The images of randomly chosen fields from each slide were captured with the digital imaging equipment, and the amount of area in each field occupied by FN fibrils was quantified as described in “Materials and Methods.” The images of randomly chosen fields from each slide were analyzed for each column in the graph. Statistical analysis was performed by using Student’s t test. **P<.0001 vs 11c.

Effect of III1-C on FN synthesis. HUASMCs were cultured on dishes that were either uncoated (control) or coated with FN. Cells on FN-coated dishes were cultured in the presence of either 50, 250, or 500 μg/mL mAb L8. After 4 days, the rate of DNA synthesis was measured by a 2-hour pulse labeling with [3H]thymidine, as described in “Materials and Methods.” The data represent the mean and standard deviation of triplicate cultures for each condition. Statistical analysis was performed by using Student’s t test. *P<.05 and **P<.01 vs control.

Treatment of HUASMCs with III1-C did not result in an immediate inhibition of DNA synthesis; the effect was not seen with treatment times of <5 hours but was seen with ≥24 hours of treatment (Fig 2). One possible explanation for this is that the cell populations that are being treated with III1-C are growing and are not synchronized in the cell cycle at the time of addition of III1-C. Previous work has shown that the ECM exerts its effects on the proliferation of fibroblasts at the G1- to S-phase transition in the cell cycle. If the same is true for III1-C treatment of VSMCs, then the only cells that will be affected immediately after the addition of III1-C are the cells that are just entering S phase; this is likely to be only a small proportion of the total cell population. The work with fibroblasts has shown that after entering S phase, cells no longer require attachment to the ECM to complete the cell cycle. Although III1-C does not cause cells to detach from the ECM, it is possible that it does exert its effects in a cell cycle–specific manner as described above. If this is true, then cells that entered S phase before the addition of III1-C may not be affected; they may continue to synthesize DNA at a normal rate. Only after their daughter cells have traversed the cell cycle and come to the next G1- to S-phase transition will III1-C inhibit DNA synthesis in these cells. Thus, the effect of III1-C on DNA synthesis may be seen to increase over time because more and more cells have come to the critical part of the cell cycle in which they are susceptible to control of DNA synthesis by III1-C. Future experiments with synchronized cell populations should help test this hypothesis for the effect of III1-C on VSMC growth.

In the present study, III1-C inhibited the FN matrix assembly of every cell type tested (rat aortic SMCs, HUASMCs, A7r5 cells, IMR-90 cells, and HUVECs); however, III1-C inhibited the growth of only the normal diploid VSMCs. These findings can be interpreted in either of two ways: (1) of the cell types tested, only VSMCs depend on an intact FN matrix for proliferation, or (2) the inhibition of VSMC growth by III1-C is due to some other effect of III1-C on VSMCs (an effect that is thus far specific for primary VSMCs) and is not a result of the inhibition of FN matrix assembly. III1-C did not cause cells to detach from the ECM; the ECM was therefore intact. Thus, the growth of only the normal diploid VSMCs is inhibited by III1-C. These findings suggest that the inhibition of VSMC growth by III1-C is due to an effect of III1-C on VSMC proliferation that is independent of any effect of III1-C on FN matrix assembly.

**Figure 7.** Quantification of effect of III1-C (C) on FN matrix assembly. Rat aortic SMCs were cultured in the presence of C or III1-C (11c) as described in the legend to Fig 6. After 3 days in culture, the cells were fixed and processed for indirect immunofluorescence with polyclonal anti-FN antibodies as described in “Materials and Methods.” The images of randomly chosen fields from each slide were captured with the digital imaging equipment, and the amount of area in each field occupied by FN fibrils was quantified as described in “Materials and Methods.” Over 20 fields from two slides were analyzed for each column in the graph. Statistical analysis was performed by using Student’s t test. **P<.0001 vs 11c.

**Figure 8.** Effect of III1-C on FN synthesis. HUASMCs were cultured in the presence or absence of III1-C or III1-C as described in the legend to Fig 1, except that cells were cultured on 10-cm dishes. After 24 hours, cells were labeled for 30 minutes at 37°C with 200 μCi of [35S]-translabel. RIPA lysates were equalized for total TCA-precipitable counts, and samples were immunoprecipitated with either preimmune IgG (lane labeled −) or anti-FN IgG (lanes labeled +). The immunoprecipitated proteins were resolved by SDS-PAGE under reducing conditions and visualized with a PhosphorImager (Molecular Dynamics, 18-hour exposure). Numbers to the left of the gel image indicate the migration locations of molecular mass markers. The area of migration of dimeric FN is indicated by the arrow labeled FN.

**Figure 9.** Inhibition of HUASMC DNA synthesis by mAb L8. HUASMCs were cultured on dishes that were either uncoated (control) or coated with FN. Cells on FN-coated dishes were cultured in the presence of either 50, 250, or 500 μg/mL mAb L8. After 4 days, the rate of DNA synthesis was measured by a 2-hour pulse labeling with [3H]thymidine, as described in “Materials and Methods.” The data represent the mean and standard deviation of triplicate cultures for each condition. Statistical analysis was performed by using Student’s t test. *P<.05 and **P<.01 vs control.

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assembly by III,-C. The results obtained with the mAb L8 are consistent with the idea that inhibition of FN matrix assembly leads to inhibition of VSMC growth. Thus, it may be that normal diploid VSMCs are acutely sensitive to the presence of a FN matrix in their environment, whereas the other cell types tested do not require the FN matrix for growth. In any case, it is clear that III,-C exerts some effect that is specific to normal VSMCs; this unique ability may prove useful in clinical applications (see below).

The mechanism(s) of how inhibition of FN matrix assembly may lead to inhibition of VSMC DNA synthesis was not addressed in the present study, but several possible mechanisms can be envisioned. For example, it is possible that the fibrillar matrix form of FN can mediate specific signals that are not generated by dimeric FN. Experiments with CHO cell adhesion and migration support this idea.55 It is known that cell shape can exert a profound effect on the proliferation of various cells.36–52 Perhaps fibrillar FN induces a cellular morphology that is more conducive to growth than nonfibrillar FN. Although we were not able to detect significant differences in cell morphology after treatment with III,-C (particularly at treatment times of \(<48\) hours), it is possible that more subtle changes do occur and that these lead to the inhibition of growth. Another possible mechanism is that the FN matrix (and its associated molecules) may serve as a reservoir for inhibitory growth factors (eg, transforming growth factor-\(\beta\)) and that the lack of a FN matrix may result in greater exposure of the cells to these inhibitory growth factors. The opposite may also be true; the FN matrix may serve as a reservoir or cofactor for stimulatory growth factors. Subsequent studies will be aimed at distinguishing between these hypotheses.

Our results demonstrated that III,-C inhibited the growth of primary rat aortic SMCs but did not inhibit the growth of the rat aortic SMC line A7r5. Primary rat aortic SMCs and A7r5 cells share many similarities, including the expression of many VSMC-specific proteins. However, A7r5 and primary cells do show differences in the expression of some genes, eg, the transcription factor GATA-6.37 In addition, the A7r5 cell line is immortalized, indicating that the normal cell cycle controls that cause primary cells to senesce are disrupted in A7r5 cells. Our results indicate that A7r5 cells have lost their dependence on the presence of a FN matrix for growth. This loss of responsiveness to the ECM may be one fundamental difference between A7r5 cells and primary rat aortic SMCs and may relate to the immortalized growth of A7r5 cells.

The properties described here for III,-C make it a potentially attractive tool for use in the treatment of VSMC proliferative diseases, such as restenosis. Balloon angioplasty destroys the endothelial lining in the blood vessel and promotes the proliferation of VSMCs, which reduces the diameter of the vessel lumen.8,4,54–56 An ideal treatment for this process of restenosis would allow endothelial cells to proliferate and migrate back into the injured area, thereby reestablishing the antithrombogenic lumen wall, but would inhibit the proliferation of VSMCs so as to prevent a reduction of the lumen diameter. III,-C was found to inhibit the proliferation of VSMCs but not of several other cell types, including endothelial cells. Moreover, III,-C exerts its effects by being applied extracellularly; it does not need to be expressed by a cell in order to produce its effects. It is therefore attractive to speculate that III,-C (or a reagent with similar properties) may serve as a useful treatment for restenosis.

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