

INTERFERON  $\gamma$  INHIBITS BOTH PROLIFERATION AND  
EXPRESSION OF DIFFERENTIATION-SPECIFIC  
 $\alpha$ -SMOOTH MUSCLE ACTIN IN ARTERIAL  
SMOOTH MUSCLE CELLS

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Muscle cell differentiation is characterized by the synthesis of contractile proteins, their organization in specialized structures, the synthesis of enzymes of specific metabolic pathways, and the inhibition of cell proliferation (1). Activation of muscle-specific gene expression is coupled to withdrawal from the cell cycle; therefore, much interest has been focused on the role of growth factors in the control of muscle differentiation (2-7). In skeletal muscle myoblasts, a reduction of growth factor concentration causes a stop of DNA synthesis, withdrawal from the cell cycle, expression of muscle-specific genes, and fusion of the myoblasts into multinucleated myotubes (7).

For smooth muscle cells (SMC)<sup>1</sup>, the process is less distinctive on the cellular level, since fusion never occurs. However, during the development of the arterial tree, the acquisition of differentiated features by SMC coincides with a decreased and/or arrested replicative activity (8). Moreover, a reduction of the concentration of serum added to SMC cultures leads to a partial reexpression of muscle-specific mRNAs (9) and of the smooth muscle-specific isoform of actin protein,  $\alpha$ -SM actin (10, 11). Similarly, an increase in serum concentration is followed by downregulation of  $\alpha$ -SM actin expression and induction of cell proliferation (10-12). This establishes a negative relationship between exposure to growth factors and differentiation, but it is unclear to what extent growth-inhibitory macromolecules affect differentiation of SMC.

Interferons (IFNs) are important inhibitors of cell proliferation, and different types of IFNs may be involved in both paracrine and autocrine growth regulation (13). IFN- $\gamma$  is produced by activated T lymphocytes, and is released during the immune response and in inflammatory conditions (13, 14). Arterial SMC respond to IFN- $\gamma$  by expression of class II MHC genes such as HLA-DR (15). These genes are expressed by SMC in the vicinity of T cell infiltrates in experimentally injured arteries

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<sup>1</sup> Abbreviation used in this paper: SMC, smooth muscle cells.

and human atherosclerotic plaques, and this is probably due to a paracrine secretion of IFN- $\gamma$  (15–18). This lymphokine is also a potent inhibitor of cell proliferation in SMC cultures (18), and may be important as a negative growth regulator in vascular lesions (18).

These observations raised the question as to whether IFN- $\gamma$  might also affect the differentiation of SMC. One could, for example, hypothesize that the inhibition of cell proliferation would be accompanied by a differentiation of SMC. We have therefore analyzed the effects of IFN- $\gamma$  on the expression of the  $\alpha$ -SM actin gene. We found that IFN- $\gamma$  inhibited the expression of  $\alpha$ -SM actin at the mRNA level in parallel with its inhibition of cell proliferation. Therefore, cessation of growth and expression of differentiation markers can be dissociated in SMC.

### Materials and Methods

**Reagents.** Rat rIFN- $\gamma$  was kindly provided by Dr. Peter van der Meide, TNO Primate Center, Rijswijk, The Netherlands. It was produced by CHO cells transfected with the chromosomal IFN- $\gamma$  gene of the rat (19, 20). Polymyxin B and cycloheximide was purchased from Sigma Chemical Co., St. Louis, MO.

**Cells.** SMC were isolated from the aorta of 6-wk-old Sprague-Dawley rats by collagenase digestion as described (9, 18) and grown in RPMI 1640 medium with 10% FCS, penicillin, and streptomycin (Gibco Laboratories, Grand Island, NY). Primary cultures, and SMC grown to passage 5 were used at subconfluent densities. They were treated with rat rIFN- $\gamma$  by addition to the medium for time intervals indicated in Results. Some cultures were also treated with cycloheximide (10  $\mu$ g/ml) by addition to the medium. In some experiments, subconfluent cultures were first synchronized in the G<sub>0</sub> phase of the cell cycle by incubation for 48 h in medium containing 0.5% FCS, and then induced to enter the cycle by addition of 10% FCS in the presence or absence of IFN- $\gamma$  and/or cycloheximide. IFN- $\gamma$  at 100 or 1,000 U/ml resulted in maximal effects (see Results), and therefore, one of these concentrations was used in experiments that did not include dose-response titrations.

**RNA Isolation.** 10<sup>7</sup> cells in 80-cm<sup>2</sup> flasks were rinsed in PBS (150 mM NaCl, 10 mM phosphate buffer, pH 7.2), detached by trypsinization, and homogenized with a Dounce glass homogenizer in 2.5 ml 6 M guanidine isothiocyanate, 0.1 M  $\beta$ -mercaptoethanol, 5 mM sodium citrate, pH 7.0, 0.5% *N*-lauryl sarcosine (21, 22). RNA was isolated by ultracentrifugation on 4 ml 5.7 M CsCl, 0.1 M EDTA, pH 7.4, as described (22), dissolved in 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and stored at -70°C until analyzed.

**RNA Analysis.** Three different hybridization probes were used for the detection of actin mRNAs. The plasmid pRA $\alpha\alpha$ -C contains a 320-bp DNA fragment corresponding to amino acids 185–291 of  $\alpha$ -actin inserted in the antisense direction in pSP65 and hybridizes to both  $\alpha$ -,  $\beta$ -, and  $\gamma$ -actin mRNAs (9). The pRA $\alpha\alpha$ -3'UT construct carries a 130-bp insert that corresponds to the 3' untranslated region of  $\alpha$ -SM actin mRNA cloned in the antisense direction in pSP64 (9). A probe prepared from this vector hybridizes exclusively to  $\alpha$ -SM actin mRNA. Synthesis of <sup>32</sup>P-labeled RNAs complementary to the  $\alpha$ -SM actin mRNA was carried out under the conditions suggested by the enzyme manufacturer using linearized pRA $\alpha\alpha$ -C or pRA $\alpha\alpha$ -3'UT plasmid DNAs, SP6 RNA polymerase (Boehringer Mannheim, Mannheim, FRG), and <sup>32</sup>P-UTP (3,000 mCi/mmol; New England Nuclear, Boston, MA; reference 23). The p91 plasmid is a cDNA clone corresponding to mouse skeletal muscle  $\alpha$ -actin mRNA (24). p91 DNA was labeled with <sup>32</sup>P-dCTP (3,000 mCi/mmol; Amersham International, Amersham, UK) using the random primer technique (25).

5  $\mu$ g of cellular RNA was denatured in 50% formamide, separated by electrophoresis through a 1.1% agarose gel containing 0.7% formaldehyde, and transferred to Hybond-C extra nitrocellulose membranes (Amersham International) by capillary blotting. After baking at 80°C overnight, the membranes were prehybridized for 4 h at 58°C with 50% formamide, 0.8 M NaCl, 50 mM Pipes, pH 6.8, 2 mM EDTA, 0.1% SDS, 2.5 $\times$  Denhardt's solution, 100  $\mu$ g/ml denatured salmon sperm DNA, and 0.5 mg/ml yeast RNA (1 $\times$  Denhardt's solution is 200  $\mu$ g/ml

polyvinylpyrrolidone, 200  $\mu\text{g/ml}$  BSA, 200  $\mu\text{g/ml}$  Ficoll 400). Membranes were then hybridized overnight at 58°C with the appropriate cRNA probe at  $10^6$  cpm/ml in a new aliquot of prehybridization buffer. Washings were carried out in a washing apparatus (Hybaid, Teddington, UK) at a flow rate of  $\sim 30$  ml/min. Membranes were washed with 1 liter of  $3 \times \text{SSC}$  ( $1 \times \text{SSC}$  is 150 mM NaCl, 15 mM sodium citrate),  $2 \times$  Denhardt's solution at 58°C, followed by 1 liter of  $0.2 \times \text{SSC}$ , 0.1% SDS, 0.1% sodium pyrophosphate at 78°C, dried, and exposed to Kodak X-Omat AS film at  $-70^\circ\text{C}$  overnight. Autoradiograms were scanned with a densitometer (Shimadzu Corp., Kyoto, Japan) at 620 nm in the transmission mode, and the peak area was used to estimate mRNA amounts. The size of mRNA species was calculated by comparison with a 0.24–9.0 kb RNA ladder (Bethesda Research Laboratories, Gaithersburg, MD).

**Protein Electrophoresis.** Cultures were rinsed twice in PBS containing 0.5 mM  $\text{CaCl}_2$  and 3 mM  $\text{MgCl}_2$ , and collected in 1% SDS containing 20 mM dithiothreitol by scraping with a rubber policeman. The samples were immediately sonicated and boiled for 3 min. Protein concentration was determined according to Bradford (26). 40- $\mu\text{g}$  amounts of protein were separated on 10% SDS-PAGE gels (27), and stained with Coomassie blue. For quantification of total actin, gels were scanned with a computerized laser beam densitometer (Genofit SA, Geneva, Switzerland) as previously described (8). Actin isoforms were analyzed by two-dimensional electrophoresis using IEF in the first dimension (28). The pH gradient was established with 2% preblended ampholines in the pH 4.0–6.5 interval (Pharmacia LKB Biotechnology, Uppsala, Sweden), gels were loaded with 30  $\mu\text{g}$  of proteins, and focused at 1,000 V for 16 h. A 10% SDS-PAGE was used for the second dimension and the gels were stained with Coomassie blue. Quantification was performed by densitometric scanning of the stained gels (8), and the relative amounts of actin isoforms were expressed as percent of total actin.

**Analysis of Actin Protein Synthesis.** Cells ( $\sim 10^6$  cells/25-cm<sup>2</sup> flask) were treated with 100 U/ml IFN- $\gamma$  for 2 d, and incubated with 100  $\mu\text{Ci}$  <sup>35</sup>S-methionine in methionine-free medium for 4 h. The cultures were then rinsed with PBS and trypsinized. Cytoskeletal extracts were prepared by extraction with 0.5% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF, and 100  $\mu\text{M}$  leupeptin (Sigma Chemical Co.) for 5 min on ice. The insoluble extracts were treated with 25  $\mu\text{g/ml}$  DNase I in PBS with PMSF and leupeptin for 15 min at room temperature, and then solubilized in SDS sample buffer with 6.25%  $\beta$ -mercaptoethanol.  $10^5$  cpm per sample was separated on two-dimensional electrophoresis using IEF in the first direction and SDS-polyacrylamide electrophoresis in the second one (28). The gels were fixed in Coomassie brilliant blue-acetic acid/methanol, destained, rinsed in Amplify (Amersham International), and dried. Autoradiograms were made on Kodak X-Omat AS film.

**Immunofluorescence.** The murine mAb anti- $\alpha\text{SM-1}$ , which recognizes a unique epitope of  $\alpha\text{-SM}$  actin (29), was used. SMC were grown on glass coverslips, rinsed in PBS, fixed in ice-cold acetone or ethanol, preincubated with 2% normal horse serum, and then incubated with anti- $\alpha\text{SM-1}$  at a concentration of 5  $\mu\text{g/ml}$ . After PBS rinses, the cells were incubated with FITC-labeled anti-mouse IgG (Amersham International), rinsed, and mounted in PBS/glycerol with *p*-phenylenediamine.

For double immunofluorescence, cells were rinsed in PBS, and incubated with rabbit anti-total actin antibodies (29) at 80  $\mu\text{g/ml}$  and anti- $\alpha\text{SM-1}$  at 5  $\mu\text{g/ml}$ . FITC-conjugated goat anti-rabbit IgG (Nordic, Tilburg, The Netherlands) and rhodamine-conjugated goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) were used for the second step. Cells were rinsed three times with PBS between incubations.

For desmin identification, an affinity-purified rabbit anti-desmin IgG (10) was used to a concentration of 0.025  $\mu\text{g/ml}$ , followed by FITC-conjugated goat anti-rabbit IgG (Behring Werke, Marburg, FRG), diluted 1:20.

All antibodies were used at optimal dilutions as determined by checkerboard titrations. Controls of staining specificity were performed by using as first-step antibodies IgG fractions of rabbit and mouse serum at the same final concentrations as those of the specific antibodies. For quantitation of immunofluorescence, 200 cells were counted in four fields of each culture, and four cultures used in each experiment.

**Analysis of Cell Proliferation.** Cells were grown in 96-well Microtiter plates. IFN- $\gamma$  was added

during exponential growth, and the wells were analyzed for cell protein by a dye-binding method, as described (18), at various time points after addition of IFN. The plates were read in a microtiter photometer (EAR400; SLT, Salzburg, Austria). Total DNA content was determined as described by Burton (30).

**Analysis of I-A Expression.** Cells were grown in 96-well Microtiter plates, stimulated with IFN- $\gamma$ , fixed, and analyzed for cell surface expression of the rat class II MHC antigen I-A by a cell surface ELISA method (18) using the mAb OX6 (Seralab, Crawley Down, UK).

## Results

**Cell Growth.** IFN- $\gamma$  inhibited proliferation of arterial SMC as revealed by a reduction of total cell protein in the culture dishes (Fig. 1). The growth inhibition was paralleled by an induction of the class II MHC antigen, I-A (Fig. 1), and was dependent on dose and time of IFN- $\gamma$  incubation (Fig. 1).

The total DNA content was estimated in SMC cultures in which  $10^4$  cells at fifth passage had initially been plated in 35-mm Petri dishes. The cultures were treated in 5 d with 1,000 U/ml IFN- $\gamma$ , and cell numbers were calculated based on a DNA content per cell of  $\sim 6$  pg (8). Using this determination,  $1.5 \times 10^6$  cells were present in IFN- $\gamma$ -treated cultures, whereas control cultures contained  $3 \times 10^6$  cells.

**Actin and Desmin Organization.** Immunofluorescence studies using actin antibodies of different specificities were performed to evaluate IFN- $\gamma$  effects on the organization of cytoplasmic actin. Antibodies to total actin mainly decorated stress fibers (Fig. 2 a). The number of cells positive for the  $\alpha$ -SM actin antibody, anti- $\alpha$ SM-1 (Fig. 2 b), was greater in primary cultures (64%) than in SMC at passage 5 (44%). After treatment with IFN- $\gamma$ , SMC were always stained with anti-total actin antibodies (Fig. 2 c), but the number of  $\alpha$ -SM actin-positive cells decreased dramatically. Many cells were negative, particularly in SMC at passage 5 (Fig. 2 d), and positive cells were moderately or weakly stained. The proportion of positive cells in IFN- $\gamma$ -treated cultures was 35% in primary SMC and 15% in SMC at passage 5. Desmin-positive cells were present exclusively in primary SMC cultures (10). Interestingly, the percentage of desmin-positive cells (51%) was the same in untreated and IFN-treated cultures.

The effects of cell density and growth state on  $\alpha$ -SM actin were further studied in passaged cells. To evaluate different phases of the cell cycle, SMC were synchronized in G<sub>0</sub> by serum starvation and then induced to enter the cell cycle by addition of

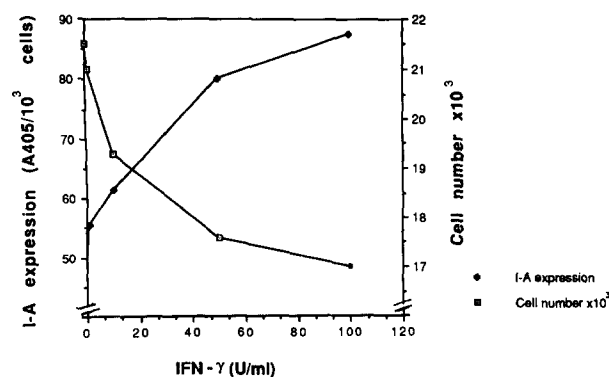
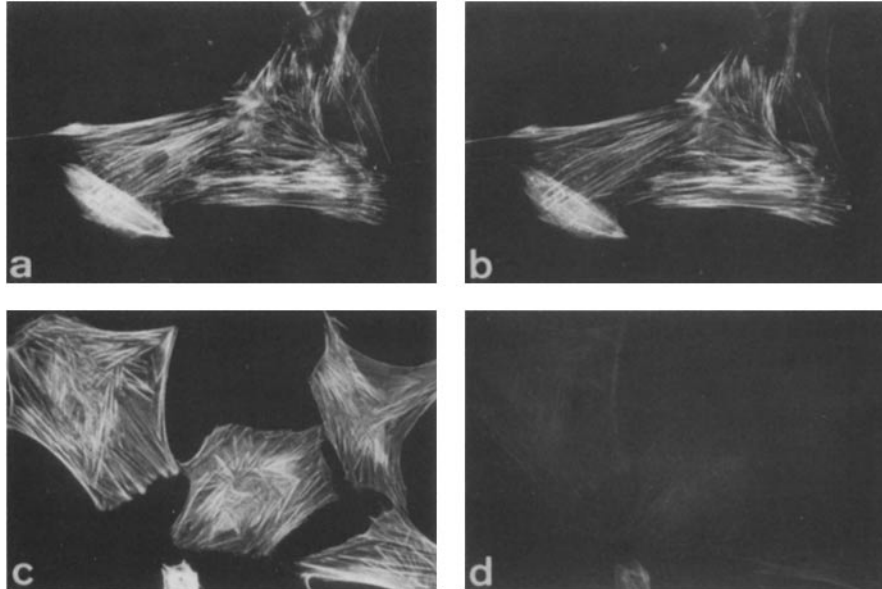


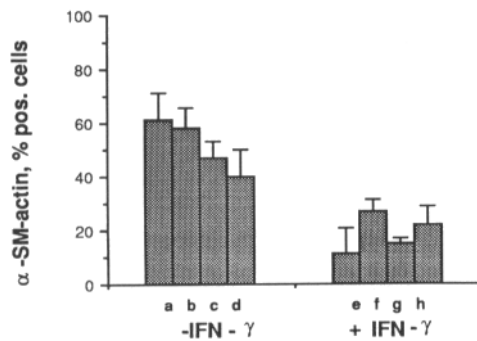
FIGURE 1. IFN- $\gamma$  inhibits proliferation and induces I-A antigen in a dose-dependent way. SMC (passage 5) were grown for 2 d in microtiter plates containing medium with IFN- $\gamma$ . Cell numbers were determined from cell protein analysis, and I-A expression from a cell surface ELISA. Coefficients of variation were  $< 2\%$ .



**FIGURE 2.** IFN- $\gamma$  selectively eliminates  $\alpha$ -SM actin stress fibers. SMC at passage 5 were grown for 5 d in regular medium (*a* and *b*) or in medium containing IFN- $\gamma$  at 1,000 U/ml (*c* and *d*). Actin was visualized by double-staining immunofluorescence using a rabbit antibody that recognizes all actin isoforms (*a* and *c*) and a mouse mAb that specifically recognizes  $\alpha$ -SM actin (*b* and *d*) ( $\times 200$ ).

serum. The frequency of  $\alpha$ -SM actin-positive cells was slightly reduced in proliferating cells when compared with quiescent ones, and the reduction was more prominent in the S and G<sub>2</sub> phases (Fig. 3).  $\alpha$ -SM actin-positive cells were significantly reduced in all IFN- $\gamma$ -treated cultures irrespective of cell cycle conditions (Fig. 3). Therefore, the reduction in  $\alpha$ -SM actin expression observed in IFN- $\gamma$ -treated cells is not secondary to cell cycle effects.

The IFN- $\gamma$ -induced reduction of  $\alpha$ -SM actin-expressing cells was (at least par-



**FIGURE 3.** The IFN- $\gamma$  effect on  $\alpha$ -SM actin is not due to cell cycle effects. SMC (passage 5) were made quiescent in 0.5% FCS for 2 d, and then stimulated to enter the cell cycle by addition of 10% FCS without (*a-d*) or with (*e-h*) IFN- $\gamma$  (100 U/ml). They were analyzed for  $\alpha$ -SM actin stress fibers by immunofluorescence at various time points corresponding to different phases in the cell cycle. Data are expressed as cells containing  $\alpha$ -SM actin stress fibers as percent of total cells (mean  $\pm$  SEM,  $n = 4$ ). (*a*) Cells in 0.5% FCS, corresponding to G<sub>0</sub>; (*b*) 12-h incubation in 10% FCS (corresponding to G<sub>1</sub>); (*c*) 24 h in 10% FCS (corresponding to S); (*d*) 36 h in 10% FCS (mainly G<sub>2</sub>); (*e*) 0.5% FCS + IFN- $\gamma$  for 36 h (i.e., in G<sub>0</sub>); (*f*) 10% FCS for 36 h, IFN- $\gamma$  added after 24 h (i.e., IFN- $\gamma$  was added during S phase); (*g*) 10% FCS for 36 h, IFN- $\gamma$  added after 12 h (i.e., IFN- $\gamma$  was added during G<sub>1</sub>); (*h*) 10% FCS and IFN- $\gamma$ , both for 36 h (i.e., IFN- $\gamma$  was added at the G<sub>0</sub>/G<sub>1</sub> transition).

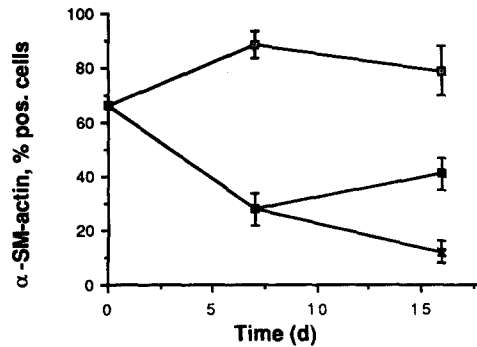


FIGURE 4. IFN- $\gamma$  effect on  $\alpha$ -SM actin is at least partially reversible. SMC (passage 5) were treated with IFN- $\gamma$  (100 U/ml) for 7 d, and then switched to fresh medium either with (X) or without (■) IFN- $\gamma$ . Controls (□) received fresh medium without IFN- $\gamma$  throughout the 14 d of the experiment. All cells were analyzed for  $\alpha$ -SM actin stress fibers by immunofluorescence. Values are percent  $\alpha$ -SM actin fiber-containing cells (mean  $\pm$  SEM,  $n = 4$ ).

tially) reversible, since withdrawal of IFN- $\gamma$  resulted in an increase in  $\alpha$ -SM actin levels towards control values (Fig. 4). It is unlikely that the effect was due to contaminating endotoxins, since addition of polymyxin B did not significantly affect  $\alpha$ -SM actin content (Fig. 5).

**Cellular Actin Content and Synthesis.** Quantitative changes in actin were examined by densitometric analysis of SDS-PAGE gels (data not shown). Actin represented 15% of total cellular protein in primary control SMC, but decreased to 8.3% after IFN- $\gamma$  treatment (1,000 U/ml for 5 d). A decrease in the percentage of actin per total protein occurred between primary culture (15%) and passage 5 (12%), and this was further decreased by IFN- $\gamma$  treatment (to 9.1%).

The proportion of  $\alpha$ -SM actin per total actin was studied by two-dimensional electrophoresis. Cultured SMC always showed a  $\beta$ -actin predominance (Fig. 6 a). After exposure to IFN- $\gamma$ , the  $\alpha$ -SM actin spot was selectively diminished both in primary cultures (Fig. 6 b) and in cultures at passage 5. Quantitative evaluation of the relative proportions of the isoforms showed that IFN- $\gamma$  treatment reduced  $\alpha$ -SM actin content both in primary and passaged cultures, with a concomitant increase in  $\beta$ - and  $\gamma$ -actin isoforms (Table I).

To better understand the effects of IFN- $\gamma$  on actin isoform expression in cultured SMC, we calculated the total protein, total actin, and single actin isoform contents as pg/cell (8) in control and IFN- $\gamma$ -treated SMC during primary cultures and at passage 5. During primary cultures, the total protein content per cell did not change in IFN- $\gamma$ -treated SMC (264 pg) compared with control SMC (262 pg). Total actin

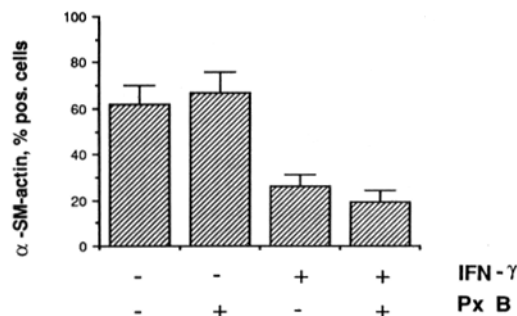


FIGURE 5. IFN- $\gamma$  effect on  $\alpha$ -SM actin is not due to endotoxin contamination. SMC (passage 5) were treated for 5 d with IFN- $\gamma$  at 100 U/ml and/or polymyxin B at 10  $\mu$ g/ml (mean  $\pm$  SEM,  $n = 4$ ).

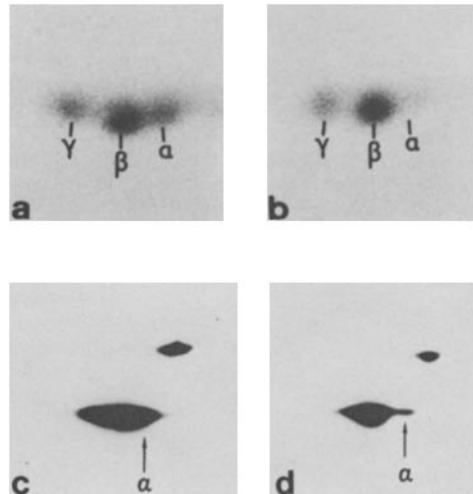


FIGURE 6.  $\alpha$ -SM actin protein content and synthesis is reduced by IFN- $\gamma$ . Protein extracts of untreated (a) or IFN- $\gamma$ -treated (1,000 U/ml, 5 d) (b) SMC were analyzed by two-dimensional electrophoresis. Untreated SMC show a predominance of  $\beta$ -actin content,  $\alpha$ -SM and  $\gamma$ -actin isoforms having Coomassie blue-stained spots comparable in size (a). In SMC treated with IFN- $\gamma$  (b), the  $\alpha$ -SM actin spot is clearly reduced. For analysis of actin synthesis, control SMC (c) and IFN- $\gamma$ -treated SMC (d) were metabolically labeled with  $^{35}\text{S}$ -methionine, and analyzed by two-dimensional electrophoresis followed by autoradiography. In IFN- $\gamma$ -treated cells, there is a clear reduction of the  $\alpha$ -SM actin spot (d).

decreased from 54.3 to 37 pg;  $\alpha$ -SM actin content decreased from 13 to 3.7 pg, and  $\beta$ - and  $\gamma$ -actins also decreased slightly (30 vs. 26 pg for  $\beta$  and 10 vs. 8 pg for  $\gamma$ ). Interestingly, in SMC at passage 5, IFN- $\gamma$  increased total protein content per cell from 262 pg in control SMC to 434 pg in IFN- $\gamma$ -treated SMC. We have no explanation for the different actions of IFN- $\gamma$  on the total protein content of primary and passage 5 SMC. Total actin increased from 31.9 to 39.7 pg; despite this slight increase,  $\alpha$ -SM actin content decreased from 3.6 to 2.6 pg while  $\beta$ - and  $\gamma$ -actins increased, respectively, from 22.3 to 29.2 pg and from 6.9 to 7.9 pg.

The synthesis of actin isoforms was analyzed by two-dimensional electrophoresis after metabolic labeling of SMC with  $^{35}\text{S}$ -methionine (Fig. 6 c). Treatment with 100 U/ml IFN- $\gamma$  for 2 d was sufficient to significantly inhibit the synthesis of the  $\alpha$ -actin isoform (Fig. 6 d).

**Actin mRNA Content.** The striking effects on actin protein synthesis and content stimulated us to further investigate the expression of the actin gene family at the mRNA level.  $^{32}\text{P}$ -labeled RNA synthesized with pRA $\alpha\alpha$ -C DNA as a template hybridizes to both  $\alpha$ -,  $\beta$ -, and  $\gamma$ -actin mRNAs, which have large sequence homologies

TABLE I  
IFN- $\gamma$  Reduces  $\alpha$ -SM Actin Protein Content

SMC culture	Treatment	Actin isoforms		
		$\alpha$	$\beta$	$\gamma$
Primary	0	24	57	19
Primary	IFN- $\gamma$	10	69	21
Passage 5	0	11	71	19
Passage 5	IFN- $\gamma$	6.6	74	20

SMC in primary culture or fifth passage were treated with IFN- $\gamma$  (1,000 U/ml, 5 d) and analyzed by densitometric scanning of two-dimensional electrophoresis gels. Values are expressed as percent of total actin content.

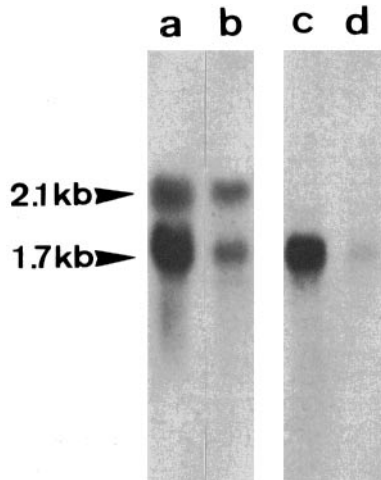


FIGURE 7. IFN- $\gamma$  reduces the steady-state level of  $\alpha$ -SM actin mRNA. Northern blot of total RNA from control (*a* and *c*) or IFN- $\gamma$ -treated (1,000 U/ml, 5 d) (*b* and *d*) SMC in primary culture, hybridized with the total actin probe PRA $\alpha\alpha$ -C (*a* and *b*) or with the  $\alpha$ -SM actin-specific probe PRA $\alpha\alpha$ -3'UT (*c* and *d*). The 2.1-kb bands correspond to cytoplasmic ( $\beta + \gamma$ ) mRNAs, while the 1.7-kb bands correspond to  $\alpha$ -SM actin mRNA. Control SMC express  $\alpha$ -SM predominance (*a*). After incubation with IFN- $\gamma$ , the 1.7-kb band is clearly reduced. A decrease in cytoplasmic RNAs is also present (*b*). Hybridization with pRA $\alpha\alpha$ -3'UT shows an almost complete disappearance of  $\alpha$ -SM actin mRNA in IFN- $\gamma$ -treated SMC (*d*) as compared with controls (*c*).

(9). Two bands of 2.1 and 1.7 kb were seen in Northern blots of total SMC RNA, corresponding to the  $\beta + \gamma$  (2.1 kb) and  $\alpha$  (1.7 kb) mRNAs, respectively (Fig. 7, lane *a*). In IFN- $\gamma$ -treated cells, the 1.7-kb band was significantly reduced, and there was also a slight reduction of the 2.1-kb band (Fig. 7, lane *b*). Similar results were obtained using the p91 total actin cDNA probe (24).

The reduction of the 1.7-kb  $\alpha$ -actin mRNA species was confirmed with the use of the  $\alpha$ -SM actin-specific cRNA probe derived from pRA $\alpha\alpha$ -3'UT. This plasmid consists of a 130-bp sequence from the 3' untranslated region of  $\alpha$ -SM actin mRNA and the cRNA probe is specific for this mRNA species (9). Fig. 7, lanes *c* and *d*, show Northern hybridization of total RNA from control SMC (lane *c*) and SMC treated with 1,000 U/ml IFN- $\gamma$  for 5 d (lane *d*).

Effects on  $\alpha$ -SM actin mRNA levels were further evaluated by densitometric scanning of Northern blots. A significant reduction of  $\alpha$ -SM actin mRNA was detectable after 12 h of treatment with IFN- $\gamma$ , and was even more striking when treatment continued for 24–72 h (Fig. 8). IFN- $\gamma$  treatment in a concentration range of 50–1,000 U/ml caused a dose-dependent inhibition of  $\alpha$ -SM actin mRNA (Fig. 9).

The IFN- $\gamma$ -induced reduction of  $\alpha$ -SM actin mRNA was abolished by cyclohexi-

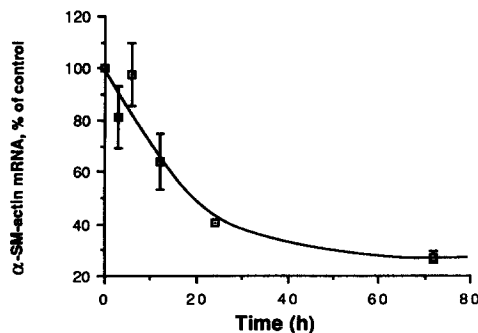


FIGURE 8. Inhibition of  $\alpha$ -SM actin mRNA is dependent on time of IFN- $\gamma$  treatment. SMC were incubated with IFN- $\gamma$  at 1,000 U/ml for 3, 6, 12, 24, or 72 h. Total RNA was separated by agarose electrophoresis in formaldehyde and  $\alpha$ -SM actin mRNA analyzed by hybridization with the  $\alpha$ -SM actin-specific probe PRA $\alpha\alpha$ -3'UT and quantitated by densitometric analysis of autoradiograms. Data are given as percent of mRNA levels in untreated control SMC (mean  $\pm$  SEM,  $n = 3$ ).



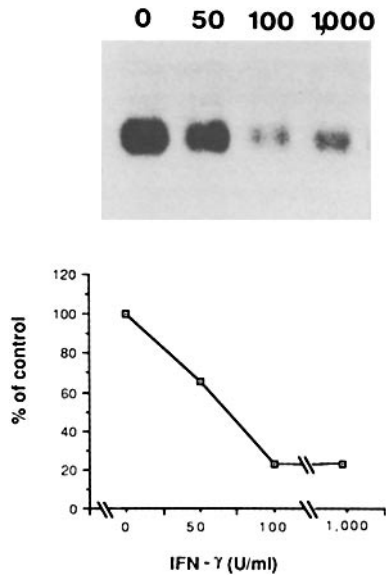


FIGURE 9. Dose dependency of IFN- $\gamma$ -induced inhibition of  $\alpha$ -SM actin mRNA. Cells were incubated for 72 h with IFN- $\gamma$  at 0, 50, 100, or 1,000 U/ml.  $\alpha$ -SM actin mRNA was analyzed by hybridization with PRA $\alpha\alpha$ -3'UT followed by densitometric scanning. Data are given as percent of mRNA levels in untreated control SMC.

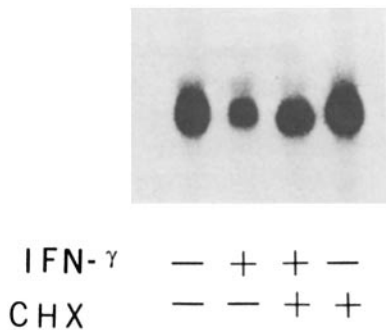


FIGURE 10. IFN- $\gamma$ -induced inhibition of  $\alpha$ -SM actin mRNA is dependent on protein synthesis. Cells were incubated for 12 h with IFN- $\gamma$  at 1,000 U/ml in the presence or absence of cycloheximide (CHX) at 10  $\mu$ g/ml. Hybridization was with the  $\alpha$ -SM actin mRNA-specific probe PRA $\alpha\alpha$ -3'UT.

mid treatment, indicating that the inhibitory effect of IFN- $\gamma$  on  $\alpha$ -SM actin gene expression was dependent on protein synthesis (Fig. 10).

### Discussion

Inflammatory mediators control phenotypic expression in the cells of the immune system. IFN- $\gamma$  upregulates or induces expression of class II MHC antigens in macrophages and other APC, and also serves as an activator of macrophages (31). It is secreted by activated T lymphocytes, and it is therefore conceivable that IFN- $\gamma$  plays an important role in the immune response.

The effects of IFN- $\gamma$  on cells not normally involved in the immune response have been more controversial. It is, however, now clear that IFN- $\gamma$  has an intrinsic antiproliferative activity, since both natural and recombinant IFN- $\gamma$  inhibits growth of many cell types, including SMC (18, 32-33). It acts by blocking the progression of the cell through the G<sub>1</sub> phase of the cell cycle (18, 34).

Recent observations suggest that IFN- $\gamma$  also affects the expression of genes not

directly related to proliferation in mesenchymal cells. We and others have shown that expression of class II MHC antigens in SMC, fibroblasts, endothelial cells, and several other types of cells is induced by IFN- $\gamma$  (16–18, 35, 36). In fibroblasts, IFN- $\gamma$  also inhibits collagen production (37–39) and the chemotactic response to fibronectin, platelet-derived growth factor, and leukotriene B4 (40).

The inhibitory effect of IFN- $\gamma$  on a specialized function of SMC and fibroblasts, such as collagen production, suggests that IFN- $\gamma$  may affect the differentiation of these cells. This possibility has now been tested and our data show that IFN- $\gamma$  inhibits the expression of smooth muscle-specific  $\alpha$ -actin. The effect is seen on both mRNA and protein levels, and our data suggest that the primary effect might be on the transcription of the  $\alpha$ -SM actin gene. This probably requires the synthesis of an inhibiting protein, since cycloheximide reversed the effect of IFN- $\gamma$  on  $\alpha$ -SM actin mRNA. An alternative explanation would be that IFN- $\gamma$  induces the synthesis of a protein that degrades  $\alpha$ -SM actin mRNA.

The inhibition of  $\alpha$ -SM actin mRNA expression induced by IFN- $\gamma$  was followed by a reduced synthesis of the  $\alpha$ -SM actin protein. The changed balance in the synthesis of actin isoforms resulted in a reorganization of the cytoskeleton, indicated by a reduced number of  $\alpha$ -SM actin-containing stress fibers in cells exposed to IFN- $\gamma$ . It appears likely that such a loss of specialized contractile structures would adversely affect the contractile capacity of SMC.

It is noteworthy that while IFN- $\gamma$  decreases  $\alpha$ -SM actin content per cell, it does not affect the number of desmin-positive cells in primary cultures, suggesting that its action is exerted specifically on actin genes but not generally on SMC cytoskeletal differentiation.

Two observations suggest that the inhibition of  $\alpha$ -SM actin expression is not secondary to the cell cycle block. First, the reduction of  $\alpha$ -actin mRNA occurred already 6–12 h after addition of IFN- $\gamma$ . Second, the effect of IFN- $\gamma$  on  $\alpha$ -SM actin stress fibers was observed in all cell cycle phases. In contrast, IFN- $\gamma$  must be added within 9 h after entry into the cell cycle in order to block DNA replication (18). Our results therefore suggest that IFN- $\gamma$  may have two independent effects: one that inhibits cell cycle progression and another one that inhibits  $\alpha$ -SM actin gene expression.

This conclusion is further supported by the observation (41) that heparin, another inhibitor of SMC proliferation, increases the level of  $\alpha$ -SM actin mRNA and protein during the arterial response to injury in vivo. Heparin, however, does not appear to act on  $\alpha$ -SM actin isoform expression per se, since its effects on actin can be explained by the block in cell cycle progression caused by this drug (41).

Previous work has shown that the control of actin isoform expression in SMC is complex; thus,  $\alpha$ -SM actin protein is less readily degraded than  $\beta$  and  $\gamma$  actin isoforms (42). Moreover, in the normal rat aorta (42) and carotid artery (41), as well as in primary cultures of SMC (9, 42), the proportion of  $\alpha$ -SM actin mRNA over total actin mRNA is significantly higher than the proportion of  $\alpha$ -SM actin protein over total actin protein synthesized in vivo. This suggests that under basal conditions, some of the  $\alpha$ -SM actin mRNA is not translated. The present results indicate that another type of control over  $\alpha$ -SM actin expression is exerted on the steady state level of mRNA, and that this level can be modulated by IFN- $\gamma$ . In myoblasts of striated muscle, TNF has been reported to exert a similar type of control

on  $\alpha$ -actin mRNA (43). This also supports the concept that cytokines of the immune system can modulate gene expression in mesenchymal cells.

Induction of differentiation is usually associated with a reduced proliferation, and vice versa, an increased proliferation is usually accompanied by a more dedifferentiated phenotype. This simple pattern does not hold true in the case of IFN- $\gamma$  stimulation of SMC, since both proliferation and expression of the differentiated phenotype are inhibited. Instead, IFN- $\gamma$  should probably be considered as a regulator of the expression of specific genes in mesenchymal cells. The combination of an inhibition of differentiated SMC properties together with an induction of class II MHC antigens makes it tempting to speculate that the net effect of IFN- $\gamma$  could be an adaptation of the SMC phenotype during local inflammatory responses.

### Summary

Differentiation of muscle cells is characterized morphologically by the acquisition of contractile filaments and characteristic shape changes, and on the molecular level by induction of the expression of several genes, including those for the muscle-specific  $\alpha$ -actin isoforms. IFN- $\gamma$  is an inhibitor of proliferation for several cells, including vascular smooth muscle, and is also an inducer of differentiated properties for several hematopoietic cells. We have therefore investigated whether IFN- $\gamma$  affects the expression of  $\alpha$ -smooth muscle actin in cultured arterial smooth muscle cells. Cells exposed to IFN- $\gamma$  show a reduction of  $\alpha$ -smooth muscle actin-containing stress fibers, as detected by immunofluorescence. The effect was observed in all phases of the cell cycle, and was caused by a reduction of the synthesis of  $\alpha$ -smooth muscle actin protein as revealed by two-dimensional electrophoretic analysis of actin isoforms. RNA hybridization using a cRNA probe that hybridizes to all actin mRNAs showed that IFN- $\gamma$ -treated cells have a reduced content of the 1.7-kb mRNA that codes for  $\alpha$ -smooth muscle actin, and to a lesser extent, also of the 2.1-kb mRNA encoding the  $\beta$  and  $\gamma$ -cytoplasmic actins. The reduction of  $\alpha$ -smooth muscle actin mRNA was confirmed using an  $\alpha$ -smooth muscle actin-specific cRNA probe. The reduction of  $\alpha$ -smooth muscle actin mRNA occurs within 12 h, and is dependent on protein synthesis, since cycloheximide treatment reversed the effect. The inhibition of this mRNA species was dose dependent, and detectable by RNA hybridization at a dose of 50 U/ml IFN- $\gamma$ . These results suggest that the differentiation of arterial smooth muscle cells is not necessarily coupled to an inhibition of cellular proliferation. Instead, IFN- $\gamma$  may regulate the expression of several genes that control both proliferation and expression of differentiation markers.

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