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INTERFERON-γ INHIBITS PROLIFERATION OF RAT VASCULAR SMOOTH MUSCLE CELLS BY NITRIC OXIDE GENERATION

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SUMMARY: Interferon(IFN)-γ inhibited the proliferation of rat vascular smooth muscle cells(VSMC) and increased the cyclic GMP(cGMP) concentration in the cells. The dose dependencies of the two effects were similar(IC_{50}=4 U/ml for the anti-proliferation and EC_{50}=3U/ml for cGMP formation) and the effect of IFN-γ was enhanced by tumor necrosis factor-α treatment. Furthermore, N^G-nitro-L-arginine, a nitric oxide(NO) synthase inhibitor, inhibited both activities induced by IFN-γ. These findings show that the anti-proliferation and cGMP formation are closely related and that IFN-γ inhibits the proliferation of rat VSMC by generation of NO through the induction of an NO synthase.

The proliferation of vascular smooth muscle cells(VSMC) is considered to be partially controlled by growth factors and cytokines(1). Interferons are known to inhibit the proliferation of various cells(2). IFN-γ has been reported to have anti-proliferative activity in VSMC in vivo and in vitro(3). VSMC respond to IFN-γ, which inhibits the proliferation of VSMC in a similar concentration manner, by expression of class II major histocompatibility complex(MHC)(4), but the relationship between anti-proliferation and expression of class II MHC has not been shown clearly. Some proteins induced by IFN-γ treatment have been suggested to be involved in the proliferation of VSMC(5).

Nitric oxide(NO) may regulate the functions of blood vessels(6), platelets(7) and brain(8), and be responsible for the cytotoxic activities of macrophages activated by cytokines(9). NO and

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Abbreviations: IFN; interferon, VSMC; vascular smooth muscle cells, MHC; major histocompatibility complex, NO; nitric oxide, sGC; soluble guanylate cyclase, NOS; NO synthase, tumor necrosis factor α; TNF-α, R-THBP; (6R)-5,6,7,8-Tetrahydro-L-biopterin, NNA; N^G-nitro-L-arginine, DMEM; Dulbecco's modified Eagle's medium, FCS; fetal bovine serum, BrdU; 5-bromo-2'-deoxyuridine, IBMX; 1-methyl 3-isobutylxanthine.

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NO-generators are thought to cause physiological responses mediated by the stimulation of the soluble guanylate cyclase(sGC) and generation of cyclic GMP(cGMP). A recent study has shown that the activity of NO synthase(NOS) is expressed constitutively in the brain and in endothelial cells(10, 11), whereas in macrophages(12, 13), and in fibroblasts(14), it is induced when activated by endotoxins or cytokines such as IFN-γ or their combination. The formation of NO by IFN-γ in macrophages or fibroblasts is enhanced in the presence of other lymphokines such as tumor necrosis factor α(TNF-α) and is dependent on (6R)-5,6,7,8-tetrahydro-L-biopterin(R-THBP)(14, 15).

In this report, we demonstrate the effect of IFN-γ on guanylate cyclase activity in rat VSMC and the relationship between the anti-proliferative activity and NO generation induced by IFN-γ.

MATERIALS AND METHODS

Materials: Recombinant human TNF-α was purified to homogeity in our laboratory(16). Recombinant rat IFN-γ was from Holland biotechnology(Netherlands). cGMP enzyme-immunoassay(EIA) kits and cell proliferation assay kits were from Amersham(U.K.). N^G-nitro-L-arginine(NNA) and other chemicals were from Sigma(MO, U.S.A.).

Culture of rat vasucular smooth muscle cells(VSMC):VSMC were derived from explants of Sprague-Dawley rats aorta(17) and maintained in Dulbecco's modified Eagle's medium(DMEM) supplemented with 10 % fetal bovine serum(DMEM/FCS). They were kept in a humidified atomosphere consisting of 90% air and 10% CO_2 at 37 °C. Cells were used for the experiments between the 5th to 8th passage.

Analysis of the proliferation of the VSMC: The proliferation of rat VSMC was estimated from their DNA synthesis. DNA synthesis in rat VSMC was assayed by measuring 5-bromo-2'-deoxyuridine(BrdU) incorporation into the cells(18). VSMC were subcultured and plated on 96-well culture dishes(10^3 cells/well) in DMEM/FCS for 2 days and to achieve quiescence, cells were then cultured in serum-free DMEM for 24 hours. Quiescent cells were incubated with IFN-γ, another compounds or both for the indicated periods(see Figures and Tables) in DMEM/FCS, followed by the addition of BrdU and incubated for further 2 hours. The BrdU incorporation in the cells was determined from the absorbance at 405 nm by using anti-BrdU based on the cell proliferation assay kits(Amersham).

Assay of cGMP levels in the VSMC:cGMP levels in the VSMC were determined by using a sensitive EIA kit(Amersham). The VSMC, plated on 24-well culture dishes(10^6 cells/well), were treated with IFN-γ, another compounds, or both for the indicated periods followed by a change to fresh DMEM containing 100 μM 1-methyl 3-isobutylxanthine(IBMX), and further 10-minute incubation at 37 °C. The reaction was terminated by addition of 0.1 N ice-cold HCl, and stored at -70 °C until analysis for cGMP by EIA.

RESULTS

We investigated the effects of IFN-γ on the proliferation of rat VSMC by analysis of the DNA synthesis determined by 5-bromo-2'-deoxyuridine(BrdU) uptake(18). Figure 1A shows the BrdU uptake of the VSMC control(open circles) and after treatment of IFN-γ(100 U/ml, closed circles) for the indicated periods. Cells were incubated for 24 hours in serum-free

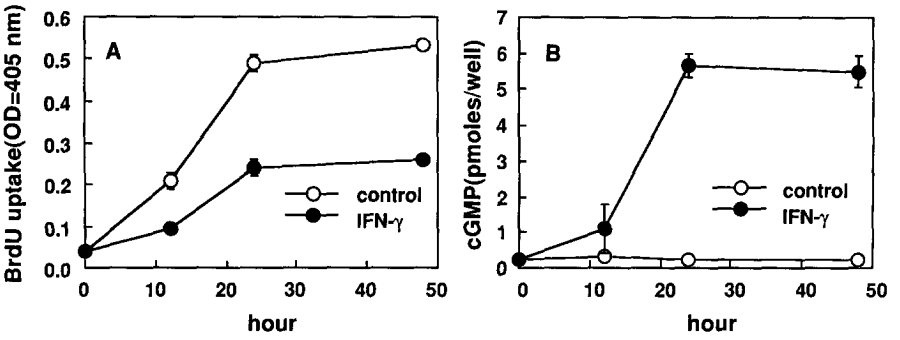

Figure 1. Time course of the DNA synthesis(A) and cGMP formation(B) in the rat VSMC in the presence of IFN-γ(100 U/ml). The proliferation of rat VSMC was estimated from the DNA synthesis determined by measuring BrdU uptake into the cells. The cGMP concentration in the VSMC was determined by EIA. VSMC were subcultured in DMEM/FCS for 2 days followed by a change to fresh FCS-free DMEM containing IFN-γ(100U/ml; closed circles) or none(opened circles), and incubation for the indicated periods. The results are expressed as the means ±SD of six and three experiments(A and B).

DMEM, followed by application of IFN-γ and change from DMEM to DMEM/FCS in this experiment. IFN-γ inhibited DNA synthesis of rat VSMC by 51.6 % for 24 hours. Figure 1B shows the cGMP formation in the rat VSMC after treatment with IFN-γ(100 U/ml) for the indicated periods as described in the above experiment. The cGMP concentration increased to 5.48 pmoles/well in the VSMC treated with IFN-γ for 24 hours. These effects were obtained only in the experiments with quiescent cells. Table I shows, in the cells treated for 24 hours with DMEM/FCS but not serum-free DMEM, the cGMP formation was increased by IFN-γ treatment

Table I. Effect of FCS pretreatment on IFN-γ activities in the rat VSMC

	hour	BrdU uptake (OD=405 nm)	cGMP (pmoles/well)
control	0	0.449±0.03	0.33±0.13
	24	0.560±0.01	0.27±0.14
IFN-γ	0	0.449±0.03	0.33±0.13
	24	0.501±0.01	7.58±1.25

The proliferation of rat VSMC was estimated from the DNA synthesis determined by measuring BrdU uptake into the cells. The cGMP concentration in the VSMC was determined by EIA. VSMC were subcultured in DMEM/FCS for 2 days followed by a change to fresh DMEM/FCS containing IFN-γ(100U/ml) or none, and incubation for 24 hours. The results are expressed as the means ±SD of six and three experiments(for BrdU uptake and cGMP levels).

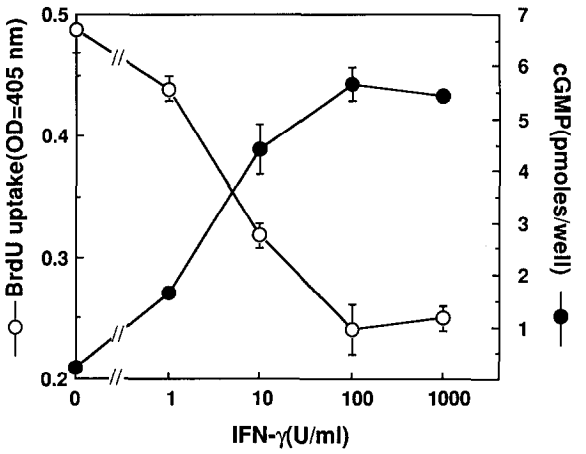

Figure 2. The relationship between anti-proliferative activity of IFN-γ in the VSMC and cGMP accumulation in the cells induced by IFN-γ. The proliferation of rat VSMC was estimated from the DNA synthesis determined by measuring BrdU uptake into the cells(opened circles). The cGMP concentration in the VSMC was determined by EIA(closed circles). VSMC were subcultured in DMEM/FCS for 2 days followed by a change to fresh FCS-free DMEM containing IFN-γ or none, and incubation for 24 hours. The results are expressed as the means ±SD of six and three experiments(for BrdU uptake and cGMP levels).

as in Figure 1B, but, the inhibition of DNA synthesis by IFN-γ was apparlently lower than that in Figure 1A.

Next, we studied the dose dependency of the two activities induced by IFN-γ. Figure 2 shows the anti-proliferative activity in the VSMC and cGMP accumulation in the cells by IFN-γ treatment. The anti-proliferative activity estimated from the inhibition of DNA synthesis occurred in a dose-dependent manner(IC_{50}=4 U/ml), which is in agreement with Hansson's data(3) in cell number. The 24-hour treatment with IFN-γ increased the cGMP concentration in the rat VSMC in a dose dependent manner up to 1000 U/ml. The estimated EC_{50} value was 3 U/ml, which is close to the IC_{50} value obtained in the DNA synthesis experiments. cGMP formation was greater in the presence of IBMX, an inhibitor of phosphodiesterase, than in the conditioned medium(data not shown).

It is well known that sGC is activated by NO. To investigate whether NO is related to the elevation of the cGMP concentration in the rat VSMC by IFN-γ treatment, we used NNA, a novel inhibitor for NOS, in the experiments shown in Table II. NNA at 1 mM inhibited not only the uptake inhibition of BrdU but also the accumulation of cGMP induced by IFN-γ in the VSMC.

Table II. Effect of NNA on IFN-γ activities in the rat VSMC

	NNA(1 mM)	BrdU uptake (OD=405 nm)	cGMP (pmoles/well)
control	-	0.488±0.02	0.23±0.09
	+	0.482±0.01	0.21±0.04
IFN-γ	-	0.241±0.02	5.66±0.33
	+	0.464±0.01	0.22±0.09

The proliferation of rat VSMC was estimated from the DNA synthesis determined by measuring BrdU uptake into the cells. The cGMP concentration in the VSMC was determined by EIA. VSMC were subcultured in DMEM/FCS for 2 days followed by a change to fresh FCS-free DMEM containing IFN-γ(100U/ml), NNA(1 mM), or both, and incubation for 24 hours. The results are expressed as the means ±SD of six and three experiments(for BrdU uptake and cGMP levels).

Not only in macrophages but also in fibroblasts, TNF-α enhances the NOS induction by IFN-γ(14, 15). We investigated the effect of TNF-α on the induction of NOS by IFN-γ. Not only the anti-proliferative activity but also the accumulation of cGMP by IFN-γ were enhanced by the presence of 50 ng/ml of TNF-α, which alone had no effect on either activities in the rat VSMC(Table III). In addition, cycloheximide, which inhibits protein synthesis, also inhibited the increase in cGMP concentration by IFN-γ treatment(data not shown).

Table III. Effect of TNF-α on IFN-γ activities in the rat VSMC

	TNF-α(50 ng/ml)	BrdU uptake (OD=405 nm)	cGMP (pmoles/well)
control	-	0.488±0.02	0.23±0.09
	+	0.453±0.02	0.29±0.11
IFN-γ	-	0.241±0.02	5.66±0.33
	+	0.172±0.01	7.87±0.30

The proliferation of rat VSMC was estimated from the DNA synthesis determined by measuring BrdU uptake into the cells. The cGMP concentration in the VSMC was determined by EIA. VSMC were subcultured in DMEM/FCS for 2 days followed by a change to fresh FCS-free DMEM containing IFN-γ(100U/ml), TNF-α(50 ng/ml), or both, and incubation for 24 hours. The results are expressed as the means ±SD of six and three experiments(for BrdU uptake and cGMP levels).

DISCUSSION

Despite data that demonstrate the anti-proliferative effect of IFN-γ on VSMC in vitro and in vivo, the detail mechanism is still unknown. Here we found that IFN-γ increases the cGMP concentration in the VSMC with the same dose dependency as anti-proliferative effect does(Figure 1 and 2), and that NNA, a potent inhibitor for NOS, completely inhibited both activities induced by IFN-γ on the VSMC(Table II). Furthermore, both IFN-γ effects were enhanced by TNF-α, which is reported to enhance NOS induction by IFN-γ in macrophages(12, 13)(Table III). These findings provide new evidence on the effect of IFN-γ on the VSMC. That is, IFN-γ inhibits the proliferation of VSMC by generation of NO through the induction of an NOS.

NO and NO-generator have been reported to have not only vasorelaxant but also anti-proliferative activities on the VSMC(19). Both activities seem to be mediated by cGMP in the VSMC because cGMP analogue can mimic NO effects on these activities(19). In addition, A- and C-type natriuretic peptides inhibited VSMC proliferation by cGMP formation(17). It should be noted that the anti-proliferative activity by cGMP depends on the cell cycle stage(3) and we obtained similar data as described in Table I. That is, using the cells treated for 24 hours with DMEM/FCS, IFN-γ increased cGMP formation, but hardly inhibited DNA synthesis.

Recently, cDNA cloning analysis has revealed the existence of at least three different speces of enzymes. Two of which are constitutive type from rat brain(20) and endothelial cells(21), and the other is an inducible type from an activated murine macrophage cell line(22, 23). Whether other types of NOS, especially inducible type of NOS, exist in other tissues remains unknown. Fleming et al.(24) and Beasley et al.(25) reported that in the VSMC, endotoxin and IL-1β also induce NOS, the activity of which depended on L-arginine but not on Ca^{2+}/calmodulin. The character of the NOS in the VSMC resembles that in activated macrophages related to their cofactor(such as R-THBP) requirements. However, both in VSMC and in macrophages, whether the same structural NOS is induced by cytokines or not remains unknown. Our findings strongly suggest that an NOS, induced by IFN-γ, is responsible for the inhibition of the proliferation of VSMC by IFN-γ treatment.

Although the relationship between in the NOS in the VSMC induced by IFN-γ and already known type remains uncertain, IFN-γ apparently induces some NOS(s), whose activity is inhibited by NNA, and that inhibits the proliferation of the VSMC. Further experiments should characterize the NOS induced by IFN-γ(or other cytokines) in the VSMC.

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