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GAMMA INTERFERON INHIBITS BASAL AND INTERLEUKIN 1-INDUCED PROSTAGLANDIN PRODUCTION AND BONE RESORPTION IN NEONATAL MOUSE CALVARIA

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Production of the osteolytic arachidonic acid metabolites, prostaglandin (PG) E_2, PGI_2 and $PGF_{2\alpha}$, by neonatal mouse calvariae was quantitated by gas chromatography/mass spectrometry. Mouse recombinant interleukin 1 (rIL-1) raised medium levels of PGE_2 and PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) in the dose range tested (1.0 - 10.0 U/ml culture medium), while an effect on $PGF_{2\alpha}$ was only observed at 10 U/ml. Bone resorption in response to rIL-1 reached a plateau at 3.0 U/ml. Mouse recombinant γ-interferon (rIFN-γ) between 100 - 500 U/ml suppressed basal PG synthesis and spontaneous resorption of cultured bone. In addition, IFN-γ at 100 U/ml prevented stimulation of PG synthesis by 3.0 U/ml rIL-1 and thereby reduced the bone resorbing activity of the cytokine by at least 60 %. 5×10^{-7} M indomethacin was equally effective in suppression of PG synthesis and bone resorption. The present study provides evidence that IFN-γ inhibits PG synthesis and consequently resorption of cultured bone.

Cells of the immune system may play a role in the regulation of bone turnover, since several of the various cytokines which they produce have been shown to differentially affect bone resorption in vitro. The monocyte/macrophage-derived interleukin 1 (IL-1), e.g., stimulated resorption in cultured neonatal mouse calvaria (1). An osteoclast activating factor (OAF), originally isolated from leukocyte cultures (3), was shown to be identical to IL-1 with respect to a long N-terminal sequence (4). In addition, an OAF-like factor distinct from IL-1 was isolated from T cell supernatants (5). Studies in our laboratory revealed that another lymphokine, γ-interferon (IFN-γ), however, inhibited resorption in bone organ cultures stimu-

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lated by various agents, such as prostaglandin E_2 (PGE_2), parathyroid hormone (6) and 1,25-dihydroxyvitamin D_3 (unpublished results). The immune interferon was particularly effective, when resorption in cultured neonatal mouse calvariae was induced by generation of endogenous PG's, whether this was due to the stimulatory action of thrombin on membrane phospholipid metabolism (7) or to utilization of exogenous arachidonic acid (8). The latter observation suggests that IFN-γ might inhibit PG synthesis through interference with the cyclooxygenase pathway of arachidonic acid metabolism. In this respect it is worthwile to note that the effects of IFN-γ on bone resorption were mimicked by indomethacin (6), a known inhibitor of cyclooxygenase activity (9).

Evidence has accumulated that the bone resorbing activity of interleukin 1, which was highly purified from monocyte conditioned media, was partially related to stimulation of PG formation (10). Recently we have shown that the stimulatory effect also of mouse recombinant interleukin 1 (rIL-1) in cultured mouse calvaria can be substantially reduced by indomethacin as well as by IFN-γ (11). However, the mechanism of action by which IL-1 and IFN-γ exert their antagonistic effects on bone resorption has still to be resolved. Recent reports by Gowen et al. (12,13) agree with our findings that IFN-γ inhibited bone resorption induced by IL-1 in cultured mouse calvaria, but failed to obtain evidence for an involvement of PG's in the action of the cytokines on bone.

Since arachidonic acid is the major precursor of PG's from membrane sites, quantitative analysis of its known osteolytic metabolites, viz. PGE_2, PGI_2 (measured as 6-keto-$PGF_{1\alpha}$), and $PGF_{2\alpha}$, by gas chromatography/mass spectrometry, as the most specific assay available, was utilized in the present study to provide direct evidence for the notion that IFN-γ reduces basal and IL-1-induced bone resorption through inhibition of PG-production in cultured bone.

Material and Methods

For bone organ culture, calvariae were dissected from 4-6 day old mice (strain HIM:OF1 Swiss, SPF, Institute for Experimental Animal Research of the University of Vienna, Himberg, Austria) and cultured as described elsewhere (14). Briefly, bones were immersed in 2.0 ml culture medium and incubated in rotating, stoppered glass tubes, which had been gassed with an $O_2/N_2/CO_2$ (50/45/5 %) mixture. Culture medium was changed after 24 h, and fresh treatments were added. Total culture period was 72 h. The culture medium was prepared from Dulbecco's modified Eagle's medium (DMEM, without L-glutamin, M.A. Bioproducts, MD) by addition of 1.4 % L-glutamin, 15 % heat-inactivated (56° C, 45 min) horse serum (Gibco, Europe), 10 U/ml heparin and 100 U/ml Na-Penicillin G. The culture medium was sterilized by filtration through 0.22 µm membrane filters. Treatments (cytokines, indomethacin) were present during the whole culture period (72 h). For quantitation of bone resorption medium calcium concentration was determined in 0.2 ml aliquots of culture medium at 0, 24, 48 and 72 h by fluorescence titration with a Corning 940 calcium analyzer.

Arachidonic acid metabolites were quantitated at the end of the second culture period: Aliquots of the culture media were adjusted to pH 3.5 with equal amounts of phosphate/citrate buffer. Proteins were denaturated by acetone. As internal standards, 20 ng/sample of each tetradeuterated $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ were then added. After extraction with ether, samples were further separated by silica chromatography, transformed into methyloxime, pentafluorobenzyl and trimethylsilyl derivatives, and finally analyzed by gas chromatography/mass spectrometry (15). The quantitation was carried out by comparing the peak areas of the labeled substance with that of the unlabeled analogue. The instrumental conditions (Finnigan 9610 gas chromatograph, Finnigan 4500 mass spectrometer) were described in detail elsewhere (16). Interassay variation (coefficient of variation) is 2.4 % for $PGF_{2\alpha}$, 2.3 % for PGE_2 and 1.4 % for 6-keto-$PGF_{1\alpha}$.

Murine recombinant gamma interferon (mIFN-γ) (specific activity 1.3 x 10^7 U/mg, Genentech, South San Francisco, CA) was obtained through Ernst Boehringer-Institut für Arzneimittelforschung, Vienna, Austria. Murine recombinant interleukin 1 (rIL-1) was a generous gift from Dr. P. Lomedico, Roche Inc., Nutley, NJ. Indomethacin was furnished by Merck, Sharp & Dohme, Vienna.

Results were tested for statistically significant differences by Student's t-test or analysis of variance, as appropriate.

Results and Discussion

As indicated by release of calcium into the medium, cultured neonatal mouse calvariae undergo spontaneous resorption to a varying degree (cf. Tab. 1-3), which has been related to the extent of endogenous PG synthesis (8,17). When in our experiments calvaria were cultured without any addition to the culture medium, they mainly produced $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, while PGE_2 was released in smaller amounts, which in some experiments were even below the detection limit of the gas chromatography/mass spectrometry assay (cf. Tab. 1-3).

It should be noted that the absolute and relative amounts of these arachidonic acid-derived PG's observed in the present study were similar to those reported previously by Voelkel et al. (18). Large interexperimental variations in PGE_2 concentrations (from 1-100 nM) had also been noted by Simmons and

Table 1. Effect of murine rIL-1 on calcium release and prostaglandin synthesis in cultured neonatal mouse calvaria

Additions to culture medium	Ca^{++} concentration (mmol/l)	PG concentration in culture medium (ng/ml)		
		PGE_2	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$
None	2.04±0.06	0.12±0.02	0.36±0.14	1.20±0.23
rIL-1 (1.0 U/ml)	2.75±0.15**	0.27±0.19	1.47±0.40	1.19±0.53
rIL-1 (3.0 U/ml)	3.18±0.10**	0.47±0.21	1.69±0.46	1.05±0.06
rIL-1 (10 U/ml)	3.10±0.10**	2.07±0.67*	4.36±0.64*	1.84±0.39

Medium calcium concentration was 1.85 mmol/l. Calvariae were precultured for 24 h, then incubated for 48 h with the same treatments. Data represent the means from 3-5 calvariae ± SEM. Asterisks indicate statistically significant difference from untreated group: * $p < 0.05$, ** $p < 0.001$ (Student's unpaired t-test).

Table 2. Inhibitory effect of murine rIFN-γ and indomethacin on calcium release and endogenous PG synthesis in cultured neonatal mouse calvaria

Additions to culture medium	Ca^{++} concentration (mmol/l)	PG concentration in culture medium (ng/ml)		
		PGE_2	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$
None	1.97±0.04	< 0.1	0.28±0.17	0.91±0.51
Indomethacin	1.57±0.03	< 0.1	< 0.1	1.00±0.29
rIFN-γ	1.48±0.02*	< 0.1	0.11±0.01	0.39±0.39
rIL-1	2.91±0.05**	0.54±0.23	1.14±0.29	0.79±0.08
rIL-1 + Indomethacin	1.95±0.06^{+}	< 0.1	< 0.1	0.46±0.26
rIL-1 + IFN-γ	1.55±0.04^{+}	< 0.1	< 0.1	0.97±0.05

For experimental conditions cf. Tab. 1. In this experiment, medium concentrations were 3.0 U/ml rIL-1, 250 U/ml IFN-γ, and $5x10^{-7}$ mol/l indomethacin. Data represent the means from 3-5 calvariae ± SEM. Analysis of variance revealed significant differences from untreated group: * $p < 0.05$, ** $p < 0.001$; from IL-1-treated group: $^{+}$ $p < 0.005$. Detection limit of PG assay: 0.1 ng/ml.

Raisz (17). From the arachidonic acid metabolites produced by cultured calvariae, PGE_2 is considered the most potent PG with respect to bone resorption, while $PGF_{2\alpha}$ has distinctly less bioactivity (19). In one study, $PGF_{2\alpha}$ even was only about 4 % as potent as PGE_2 (20). PGI_2 is believed to exhibit considerable bone resorbing activity, but its rapid conversion into inactive 6-keto-$PGF_{1\alpha}$ makes it difficult to assess its potency (19).

Table 1 shows that mouse recombinant interleukin 1 (rIL-1), in the dose range tested (1.0-10.0 U/ml), increased production of PGE_2 and of 6-keto-$PGF_{1\alpha}$, while a distinct effect on $PGF_{2\alpha}$ was observed only at 10.0 U/ml. Concomitantly, the cytokine induced bone resorption, although cultured calvariae could not respond to concentrations higher than 3.0 U/ml by further release of calcium (Tab. 1).

Table 3. Dose-dependence of the inhibitory effect of rIFN-γ on basal and rIL-1-stimulated calcium release and PG synthesis

Additions to culture medium		Ca^{++} concentration (mmol/l)	PG concentration in culture medium (ng/ml)		
			PGE_2	6-keto-$PGF_{1\alpha}$	$PGF_{2\alpha}$
rIL-1 (U/ml)	rIFN-γ (U/ml)				
-	-	2.30±0.15	< 0.1	0.92±0.57	1.13±0.01
3.0	-	3.11±0.19**	4.91±1.80**	9.21±2.17*	1.24±0.23
-	100	1.64±0.03**	< 0.1	0.14±0.04	1.31±0.20
-	250	1.50±0.02**	< 0.1	< 0.1	1.19±0.08
-	500	1.44±0.02**	< 0.1	< 0.1	1.13±0.19
3.0	100	2.22±0.09^{++}	0.33±0.19^{++}	0.28±0.18^{+}	1.33±0.36
3.0	250	1.95±0.06^{++}	0.51±0.33^{++}	< 0.1	0.80±0.05
3.0	500	2.09±0.08^{++}	< 0.1	< 0.1	1.06±0.06

For experimental conditions cf. Tab. 1.
Data represent the mean from 3-5 calvariae ± SEM. Analysis of variance revealed significant difference from untreated group: * $p < 0.05$, ** $p < 0.005$; from IL-1-treated group: $^{+}$ < 0.05, $^{++}$ $p < 0.005$. Detection limit of PG assay: 0.1 ng/ml.

In another experiment, mouse recombinant gamma interferon (rIFN-γ) was shown to inhibit both unstimulated and rIL-1-induced PG production in parallel with bone resorption (Tab. 2). Again, PGE_2 and 6-keto-$PGF_{1\alpha}$ were the arachidonic acid metabolites mainly affected. In this respect, there was no difference between the effect of rIFN-γ and of indomethacin (Table 2), whose inhibition of the cyclooxygenase pathway of arachidonic acid metabolism is well established (9).

Tab. 3 shows the dose-dependence of the inhibitory effect of rIFN-γ on PG synthesis and bone resorption induced by rIL-1. rIFN-γ at 100 U/ml prevents stimulation of PG synthesis by rIL-1 and reduces bone resorption by approximately 60 %. Treatment of calvariae with higher concentrations of the immune interferon results in further reduction of PG formation, particular in that of PGE_2 and 6-keto-$PGF_{1\alpha}$, but has no additional effect on calcium release into the medium. Thus, the bone resorbing activity of IL-1 as noted before (1,10,11) apparently is only partially mediated by PG's.

Our study clearly demonstrates that mouse rIL-1 stimulates the production of bone resorbing PGE_2, PGI_2 and $PGF_{2\alpha}$, in cultured mouse calvaria. It extends the findings of Sato et al. (21) that heterologous human rIL-1 stimulates PGE_2 production in this system. More importantly, our results provide strong evidence for the inhibitory action of IFN-γ on PG formation in bone and, consequently, on its resorption.

From the fact that IFN-γ production by T-lymphocytes can be indirectly stimulated by IL-1, namely through release of IL-2, it can be inferred that IL-1 and IFN-γ could be engaged in a feed-back regulation of bone turnover at the level of PG formation. This might be ineffective in immune-related inflammatory bone disease, such as rheumatoid arthritis, where, on the one hand, basal and mitogen-stimulated production of IFN-γ by T-lymphocytes may be impaired (22) whereas on the other hand, IL-1 induced PG-synthesis must be considered as cause of severe bone destruction. Thus, the inhibitory effect of IFN-γ on PG-synthesis in bone may provide a rationale for the use of the immune interferon in the treatment of rheumatoid arthritis and other related bone diseases.

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