Vol. 196, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1993 Pages 25-31

Growth factor mRNA profiles in unstimulated human mononuclear cells: Identification of genes which are constitutively and variably expressed

Ellen Jendraschak, Wolfgang E. Kaminski, [1] Franz Hessel,
Rosemarie Kiefl, and Clemens von Schacky [2]

Medizinische Klinik, Klinikum Innenstadt, University of Munich,
D-80336 Munich, FRG

Received August 23, 1993

Quiescent human mononuclear cells (MNC) as studied *ex vivo* express highly specific mRNA patterns of growth factors: We recently demonstrated that unstimulated MNC constitutively express the genes for the A and B chains of platelet-derived growth factor (PDGF). This expression was down-regulated by dietary ω-3 fatty acids. We now report that unstimulated human MNC express the genes for platelet-derived endothelial cell growth factor (PD-ECGF), insulin-like growth factor (IGF-1A, -1B) and transforming growth factor-β1 (TGF-β1). In contrast, acidic and basic fibroblast GF (FGFs), insulin-like GF-2 (IGF-2), transforming GF-α (TGF-α) and epidermal GF (EGF) were not expressed in MNC, nor were α- and β- receptors for PDGF. Quantitatively, as measured over a period of six weeks, expression of PD-ECGF was constant, whereas TGF-β1, IGF-1A, and IGF-1B were expressed at varying levels and all independently of each other. Dietary ω-3 fatty acids had no effect on gene expression. Our results also indicate that down-regulation of PDGF gene expression represents a specific and possibly therapeutic effect of dietary fish oil supplementation.

Growth factors such as PDGF promote mitogenesis, chemotaxis and/or differentiation of a number of cell types (1). They act alone or in concert with other growth factors (1). cDNA sequences, protein structure and biological activities of most growth factors are well characterized *in vitro*. In contrast, little is known concerning sites and control of synthesis, mechanisms of action and the interrelationships of growth factors *in vivo* (1). We recently demonstrated that unstimulated human mononuclear cells express remarkably constant mRNA levels of PDGF-A and PDGF-B under unaltered dietary conditions (2). Supplementation of the diet with ω-3 fatty acids resulted in down-

[1] Dr. Kaminski's present address is Divisions of Hematology-Oncology, Departments of Medicine and Pathology, Department of Veterans Affairs Medical Center, New York 10010; and Cornell University Medical College, New York 10021.

[2] To whom correspondence should be addressed.

0006-291X/93 $4.00

regulation of mRNA levels of PDGF-A and PDGF-B (2). mRNA was detected and quantified with 3n-PCR (a modified RNA-PCR technique, ref. 2). It is not known whether this down-regulation of PDGF mRNA is a specific effect or represents a general metabolic property of ω-3 fatty acids. Moreover, very little information is available concerning mRNA expression of growth-regulatory proteins in unstimulated MNC *in vivo* (2, 3). Therefore, we determined specific mRNA expression for other growth factors, believed to be involved in proliferation and mitogenesis of endothelial cells (EC), smooth muscle cells (SMC), and/or fibroblasts in 42 samples of unstimulated human mononuclear cells.

Materials and Methods

Details of blood cell preparation, mRNA isolation, cDNA synthesis, polymerase chain reaction, mRNA quantitation, and the volunteer study were described previously (2). In addition, we excluded confounding processed genomic sequences (4). In brief:
Isolation of MNC and granulocytes: Venous blood samples were obtained after an overnight fast. Peripheral blood cells were prepared by centrifugation through ficoll-hypaque. The platelet-free (fig. 3, ref. 5, 6) mononuclear fraction (> 95 % MNC) contained on average 60 % monocytes, 35 % lymphocytes and < 5 % granulocytes. The granulocyte fraction contained > 98% granulocytes (on average 92 % neutrophils, 7 % eosinophils, 1 % MNC). Cells were viable (>95%) and unactivated, as demonstrated by an appropriate assay for interleukin1β (2). Total RNA was isolated from aliquots of 1.0 x 10^7 MNC (4.0 x 10^7 for granulocytes).
Isolation of platelets: Human blood platelets were collected from platelet-rich plasma (5, 6) by centrifugation at 2000 x g for 15 min and washed twice with icecold phosphate-buffered saline, pH 7.4. Contaminating erythrocytes were removed by hypotonic lysis. The ratio of platelets to contaminating leucocytes was 20.000:1. Total RNA was prepared from aliquots of 2.0 x 10^9 platelets.
Preparation of total RNA, DNA and cDNA synthesis: Total RNA was extracted and reverse-transcribed into cDNA, using M-MLV reverse transcriptase according to the manufacturers' protocol (Gibco-BRL, Eggenstein, Germany). DNA was prepared accordingly, but without reverse transcription.
Polymerase chain reaction: 3n-PCR was performed in 50 µl total reaction volume containing 10 mM Tris-HCl pH 8.5, 50 mM KCl, 2.0 mM $MgCl_2$,10 mM dNTP, 1.25 units of Amplitaq DNA polymerase (Perkin-Elmer Cetus, Überlingen, FRG) and oligonucleotide primers (0.04 µM external primers, 1.5 µM internal primers). The amplification profile was: 95 °C, 45 sec; 55 °C, 60 sec; 72 °C, 45 sec. After external amplification with 25 cycles a 10 % aliquot was subjected to the second amplification (20-30 cycles) with internal primers. 20 µl aliquots of the resulting PCR reaction mixture were separated on an ethidium bromid stained agarose gel (1.5 % agarose type I, Sigma, Munich, FRG) by electrophoresis. Gels were photographed under UV light (Polaroid 665 film, Sigma, Munich, FRG), and densitrometric analysis was performed on the negatives with an UltraScan laser densitometer (Pharmacia LKB, Freiburg, FRG).
Oligonucleotide primers: 20-mer nested oligonucleotide primers were synthesized (Microgen, Munich, FRG) according to known cDNA sequences (tab. 1). As a positive control, all primers were tested using a mixture of cDNAs from the human cell lines RD and HUT 102 as template (2), which contained transcripts of all genes investigated in this study (data not shown). Primers were tested for spanning introns and for exclusion of processed genomic sequences (4) using DNA templates (not shown).
mRNA quantitation: Expression of β-actin mRNA served as internal standard for the relative mRNA quantitation of the respective genes (2). When quantifying samples, 3n-PCR analyses were performed using identical lots of reagents and stock solutions. Appropriate dilution series of the cDNA samples to be compared served to establish

standard curves for quantifications (2). All quantitations were confirmed twice and the mean determined (tab. 2).
Volunteer study: Fourteen healthy male volunteers were randomized pairwise. Seven volunteers supplemented their diet with 7 g / day of a fish oil concentrate (K85, Norsk Hydro, Porsgunn, Norway) for six weeks. The other seven served as controls. Blood was obtained on the previous day, after one week and after six weeks. At each timepoint volunteers were in good health as assessed by history and routine laboratory tests.

Results and Discussion

Unstimulated human mononuclear cells express mRNAs specific for PD-ECGF, TGF-β1, IGF-1A and IGF-1B (fig. 1,2, tab. 1,2). This is the first demonstration of mRNA expression of PD-ECGF, IGF-1A and IGF-1B in these cells. Levels of PD-ECGF mRNA were remarkably constant at all timepoints, suggesting constitutive PD-ECGF gene expression in MNC (fig.1, tab. 2). Variations up to 20% were observed in 10 of 42 timepoints (tab. 2). Only a limited number of cell types can synthesize PD-ECGF or express PD-ECGF mRNA (7). Originally purified from platelets, PD-ECGF is synthesized by human foreskin fibroblasts and some carcinoma cell-lines (7). In contrast, a variety of other cell types are unable to express PD-ECGF (7). *In vitro*, PD-ECGF stimulates proliferation and chemotaxis of endothelial cells (8). Although an *in vivo* function has not been defined, it has been speculated that PD-ECGF may be involved in smooth muscle proliferation, possibly contributing to the pathogenesis of atherosclerosis (1, 8). Whether PD-ECGF is formed by mononuclear cells, and plays a part in atherogenesis remains to be clarified.

TGF-β is a strong chemoattractant for macrophages and stimulates expression of PDGF and TGF-β itself (9). Thus it may act in an autocrine fashion for macrophages at an inflammatory site. TGF-β mRNA is also expressed in human MNC (fig. 1, ref. 10). However its expression over time has not yet been demonstrated. We found TGF-β1 was expressed at all 42 timepoints studied. Individual expression varied up to 80 % (fig. 1, tab. 2). This may reflect constitutive expression of TGF-β1 which, in contrast to the constant expression of PD-ECGF mRNA, is variable over time. Research is beginning to focus on mechanisms regulating biosynthesis of TGF-β1 on the transcriptional level (11).

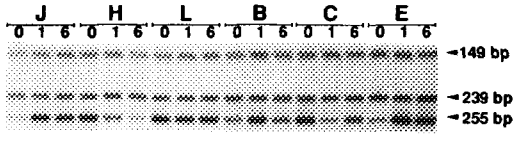

Figure 1. Expression of PD-ECGF, TGF-β1 and β-actin mRNA in mononuclear cells from 6 subjects subsisting on an unaltered diet (J, H, L) and before (0), after 1 and after 6 weeks of ω-3 fatty acid supplementation (B, C, E). After extraction of RNA and cDNA synthesis, β-actin (239 bp), PD-ECGF (149 bp) and TGF-β1 (255 bp) were amplified with 3n-PCR, analysed by gel electrophoresis and photographed under UV-light as indicated above (Materials and Methods).

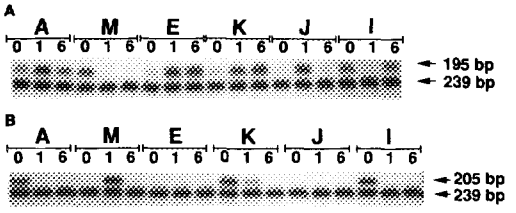

Figure 2. Expression of IGF-1A (A) and IGF-1B (B) mRNA in MNC from 6 subjects before (0), after 1 week and after 6 weeks of supplementation with dietary ω-3 fatty acids (A, E, J) and with an unaltered diet (M, K, I). IGF-1A (195 bp), IGF-1B (205 bp) and β-actin were amplified with 3n-PCR and further analysed as described in fig 1.

IGF-1 stimulates proliferation of MNC, among other cells (12). Human alveolar macrophages express two different IGF-1 mRNAs, termed IGF-1A and IGF-1B. Both are transcribed from a single gene, with a higher expression rate of IGF-1A than -1B (13). Although IGF-1 may be secreted from monocytes (12), expression of IGF-1 mRNA has not as yet been reported in human MNC. With our highly sensitive 3n-PCR method, we detected IGF-1A and IGF-1B mRNA in unstimulated MNC from 13 of 14 individuals. Relative mRNA levels of IGF-1A and IGF-1B varied widely intra- and interindividually (fig. 2, tab. 2). At 10 timepoints no expression was detected. The variations of IGF-1A and -1B did not occur in parallel, indicating different regulation pathways for both transcripts (fig. 2, tab. 2). IGF-1A mRNA was detected at 30 timepoints, IGF-1B was expressed at 10 timepoints, whereas IGF-1A and -1B together were expressed at 8 timepoints. In rat liver *in vivo* (14), and in rat adipocytes *in vitro* (15), expression of different IGF-1 mRNA species is regulated by growth hormone (GH). Regulation of different IGF-1 mRNAs in humans is essentially unknown (13). In humans GH is secreted in a pulsatile manner (16). Our data do not rule out an effect of intermittent GH secretion on mRNA expression of at least one of the IGF-1 mRNA transcripts in human mononuclear cells.

Table 1. Characterization of specific amplification products. Diagnostic segments for each growth factor representing positions and lengths (bp) indicated were amplified with internal nested primers. External primers were located next to internal primers, at a distance of one single base. Each segment contained a characteristic restriction enzyme site (RE) for identification. Specific RE cleavage sites (CS) are shown. Position numbers refer to published cDNA sequences (18-25). In human mononuclear cells specific mRNA expression was detected for the genes indicated with "+".

	position	bp	RE	CS	mRNA
β-actin	810-1048	**239**	*HinfI*	850	+
TGF-β1	1711-1965	**255**	*SmaI*	1886	+
IGF-1B	2243-2683	**205**	*Sau3A*	2312	+
IGF-1A	2243-3664	**195**	*RsaI*	3650	+
PD-ECGF	779-927	**149**	*SmaI*	868	+
EGF	3794-3975	**203**	*AluI*	3927	-
TGF-α	256-535	**280**	*EaeI*	297	-
aFGF	58-238	**181**	*BamHI*	122	-
bFGF	58-277	**220**	*EaeI*	148	-
IGF-2	73-274	**202**	*PvuII*	221	-
PDGF β-Rc	3005-3202	**198**	*PvuII*	3045	-
PDGF α-Rc	3163-3374	**212**	*AluI*	3250	-

Table 2. Quantitative analyses of mRNA expression of PD-ECGF, TGF-β1, IGF-1A and IGF-1B in mononuclear cells before (0), after 1 week and after 6 weeks of dietary ω-3 fatty acids (A-G) compared with an unaltered diet (H-N). Data were obtained from 14 subjects (42 timepoints). mRNA quantities for each subject were normalized to expression of β-actin. Values are indicated as multiplies of 0.2 and represent quantities of mRNA relative to the maximum value for each subject, which was set to 1.0.

ω-3 fatty acids	A			B			C			D			E			F			G		
weeks	0	1	6	0	1	6	0	1	6	0	1	6	0	1	6	0	1	6	0	1	6
PD-ECGF	0.8	1.0	0.8	1.0	1.0	1.0	0.8	1.0	0.8	1.0	0.8	0.8	1.0	1.0	0.8	1.0	1.0	1.0	1.0	1.0	0.8
TGF-β1	1.0	1.0	1.0	0.4	1.0	0.4	1.0	0.2	0.4	0.2	0.4	1.0	0.2	1.0	0.8	1.0	1.0	1.0	1.0	1.0	0.6
IGF-1A	0.4	1.0	0.6	0.2	1.0	0.8	0.2	1.0	0.2	1.0	0	1.0	0.2	1.0	0.8	1.0	0	0	1.0	0.8	0
IGF-1B	1.0	0	0	0	0	0	1.0	0	0	0	0	0	0	0	0	0	1.0	0	1.0	0	0

regular diet	H			I			J			K			L			M			N		
weeks	0	1	6	0	1	6	0	1	6	0	1	6	0	1	6	0	1	6	0	1	6
PD-ECGF	1.0	1.0	0.8	1.0	1.0	1.0	0.8	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
TGF-β1	1.0	0.4	0.2	1.0	0.8	0.6	0.2	1.0	0.8	1.0	0.4	0.2	1.0	0.8	1.0	1.0	1.0	0.2	1.0	0.4	0.8
IGF-1A	0.2	0.2	1.0	1.0	0.2	0.8	0	1.0	0	0.2	0.8	1.0	0	0	0	1.0	0	0	1.0	0	0.8
IGF-1B	1.0	0	0	1.0	0	0	0	0	0	1.0	0.4	0	0	0	0	0	1.0	0	0	0	1.0

In contrast to activated monocytes/macrophages (9), mRNA specific for TGF-α and bFGF was not detected in unstimulated cells (not shown). Furthermore, mRNAs of EGF (fig. 3), aFGF, IGF-2 and PDGF-receptor α and β were not detected at any of the 42 timepoints (not shown). Our cell preparation contained on average 60% monocytes, 35% lymphocytes, < 5% granolocytes, and no platelets (fig. 3). 3n-PCR is a highly sensitive method for the detection of specific mRNA (2). We did not detect mRNA for any of the growth factors in unstimulated granulocytes either (not shown). Therefore, our data indicate that unstimulated leukocytes do not express genes for TGF-α, bFGF, aFGF, IGF-2 and EGF. Moreover, the absence of PDGF-receptor α and β mRNA, suggests that any PDGF protein synthezised in unstimulated MNC may not act in an autocrine manner. This is supported by data obtained from activated MNC in atherosclerotic lesions (17).

Expression levels of all the growth factor genes reported herein were studied in volunteers who supplemented their diet with ω-3 fatty acids for 6 weeks. No differences in expression levels of any of the above growth factor genes were detected in control subjects. We previously described lowering of PDGF-A and -B mRNA levels by dietary

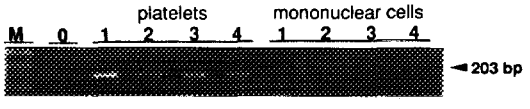

Figure 3. Detection of EGF mRNA expression (203 bp) in human platelets (lanes 1-4, left) from 4 different donors, in contrast to unstimulated mononuclear cells, which do not express EGF(lanes 1-4, right). (0) negative control, (M) DNA size marker (pBR328/*HaeIII*). Our observations indicate that EGF mRNA can serve as a marker for verifying the presence of platelets. Therefore the absence of EGF mRNA in all of our mononuclear cell preparations strongly indicated that the mRNAs detected for PD-ECGF, TGF-β1, IGF-1A and IGF-1B did not originate from platelets.

ω-3 fatty acids (2). Lowering of PDGF-A and -B mRNA levels thus apparently represents a specific biological/ biochemical effect of fish oil ingestion and not a general down-regulation phenomenon.

Our results indicate that circulating unstimulated MNC express a unique pattern of growth factor genes. Some of these, including PDGF-A, PDGF-B, PD-ECGF and TGF-β1 are constitutively expressed. Constant gene expression for PDGF-A, PDGF-B (2) and PD-ECGF suggests basal expression of these growth-regulatory molecules. In contrast, genes for other growth factors, like TGF-β1 and IGF-1, are expressed at varying levels. Factors modulating such variable expression *in vivo* in human MNC remain to be identified.

Acknowledgments

Supported by the Deutsche Forschungsgemeinschaft (Scha 398). Norsk Hydro provided K 85. We thank AJ Marcus and MJ Broekman for discussing the manuscript.

References

1. Ross, R. (1993) Nature, 362, 801-809
2. Kaminski, W. E., Jendraschak, E. Kiefl, R. and von Schacky, C. (1993) Blood, 81, 1871- 1879
3. Rappolee, D. A., Mark, D., Banda, M. J. and Werb, Z. (1988) Science, 241, 708-12
4. Potten, H., Jendraschak, E., Hauck, S., Amar, L. C., Avner, P. and Müllhofer, G. (1988) Gene, 71, 461-471
5. von Schacky, C., Kiefl, R., Jendraschak, E. and Kaminski, W. E. (1993) J. Lab. Clin. Med., 121, 302-309
6. von Schacky, C., Marcus, A. J., Safier, L. B., Ullman, H. L., Islam, N., Broekman, M. J. and Fischer, S. (1990) J. Lipid. Res., 31, 801-810
7. Usuki, K., Heldin, N. E., Miyazono, K., Ishikawa, F., Takaku, F., Westermark, B. and Heldin, C. H. (1989) Proc. Natl. Acad. Sci. U S A, 86, 7427-7431
8. Ishikawa, F., Miyazono, K., Hellman, U., Drexler, H., Wernstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W. and Heldin, C. H. (1989) Nature, 338, 557-562
9. Rappolee, D. A. and Werb, Z. (1992) Curr. Top. Microbiol. Immunol., 181, 87-140
10. Assoian, R. K., Fleurdelys, B. E., Stevenson, H. C., Miller, P. J., Madtes, D. K., Raines, E. W., Ross, R. and Sporn, M. B. (1987) Proc. Natl. Acad. Sci. U S A, 84, 6020-6024
11. Sporn, M. B. and Roberts, A. B. (1992) J. Cell. Biol., 119, 1017-1021
12. Kooijman, R., Willems, M., Rijkers, G. T., Brinkman, A., van Buul-Offers, S. C., Heijnen, C. J. and Zegers, B. J. M. (1992) J. Neuroimmunol., 38, 95-104
13. Nagaoka, I., Someya, A., Iwabuchi, K. and Yamashita, T. (1991) FEBS Lett., 280, 79-83
14. Foyt, H. L., Lanau, F., Woloschak, M., LeRoith, D. and Roberts, C. T. Jr. (1992) Mol. Endocrinol., 6, 1881-1888
15. Doglio, A., Dani, C., Fredrikson, G., Grimaldi, P. and Ailhaud, G. (1987) EMBO J., 6, 4011-64016
16. Zadik, Z., Chalew, S. A., McCarter, R. J., Meistas, M. and Kowarski, A. A. (1985) J.Clin. Endocrinol. Metab., 60, 513-516
17. Wilcox, J. N., Smith, K. M., Williams, L. T., Schwartz, S. M. and Gordon, D. (1988) J. Clin. Invest., 82, 1134-1143

18. Ponte, P., Ng, S. Y., Engel, J., Gunning, P. and Kedes, L. (1984) Nucleic Acids Res., 12, 1687-1696
19. Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., Assoian, R. K., Roberts, A. B., Sporn, M. B. and Goeddel, D. V. (1985) Nature, 316, 701-705
20. Rotwein, P., Pollock, K. M., Didier, D. K. and Krivi, G. G. (1986) J. Biol. Chem., 261, 4828-4832
21. Bell, G. I., Fong, N. M., Stempien, M. M., Wormsted, M. A., Caput, D., Ku, L. L., Urdea, M. S., Rall, L. B. and Sanchez, P. R. (1986) Nucleic Acids Res., 14, 8427-8446
22. Derynck, R., Roberts, A. B., Winkler, M. E., Chen, E. Y. and Goeddel, D. V. (1984) Cell, 38, 287-297
23. Mergia, A., Tischer, E., Graves, D., Tumolo, A., Miller, J., Gospodarowicz, D., Abraham, J. A., Shipley, G. D. and Fiddes, J. C. (1989) Biochem. Biophys. Res. Commun. 164, 1121-1129
24. Abraham, J. A., Whang, J. L., Tumolo, A., Mergia, A., Friedman, J., Gospodarowicz, D. and Fiddes, J. C. (1986) Embo J., 5, 2523-2528
25. de Pagter-Holthuizen, P., van Schaik, F. M., Verduijn, G. M., van Ommen, G. J., Bouma, B. N., Jansen, M. and Sussenbach, J. S. (1986) FFBS Lett., 195,179-184