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Expression of GM-CSF and a Functional GM-CSF Receptor in the Human Colon Carcinoma Cell Line SW403

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SUMMARY: Only little is known about the expression of a functional granulocyte-macrophage colony-stimulating factor receptor (GMR) and its ligands in human colon carcinoma cell lines. To obtain more information on this subject, we investigated the human colon carcinoma cell line SW403, and we were able to demonstrate the constitutive synthesis of a 85-kDa GMR in SW403 cells. After stimulation with 10% fetal calf serum, GM-CSF transcripts were expressed as well. The incubation of SW403 cells with GM-CSF resulted in an intensive down-regulation of the activity of the interferon-γ-receptor gene, which could be reconstituted by simultaneous addition of an anti-GM-CSF antibody. GM-CSF induced an activation of tyrosine-phosphorylated protein kinases with molecular weights in the range of 30 to 210 kDa, but it had no effect on the DNA synthesis of SW403 cells.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine which stimulates myeloid bone marrow progenitor cell proliferation and differentiation and enhances the effector function of mature neutrophils and monocytes (1, 2). GM-CSF exerts its biological function by acting on the GM-CSF receptor (GMR) complex, a heterodimer consisting of an α chain (p85) and a β_c chain (gp120). Being a member of the hematopoietin receptor superfamily, GMR has consequently no intrinsic tyrosine kinase activity. Stimulation of myeloid cell lines with GM-CSF induces rapid protein tyrosine phosphorylation indicating, that protein tyrosine kinases may be associated with the GMR and that they are components of the GM-CSF-activated signal transduction pathways (3, 4).

Although some cell lines from non-hematopoietic tumors can respond to GM-CSF (5, 6), the expression of functional GM-CSF receptors is largely limited to hematopoietic cells. GM-CSF-induced signal transduction pathways in non-hematopoietic cells are only poorly understood, and the function of GMR in these cells is unknown. In the present study, we looked for GM-CSF and GMR expression

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in the human adenocarcinoma cell line SW403 and examined effects of GM-CSF on modulation of gene expression, cell growth and activation of phosphotyrosine protein kinases. We report that GM-CSF and a functional GMR are expressed in SW403 cells, and that GM-CSF induces activation of various kinases but has no influence on SW403 cell growth.

MATERIALS AND METHODS

SW403 colon adenocarcinoma cells and cell culture conditions. The SW403 cell line was obtained from the American Type Culture Collection (Rockville, Md.) and originated from a human adenocarcinoma of the colon (Leibovitz et al. 1976). Cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (FCS), L-glutamine (2 mmol/l), penicillin (100U/ml) and streptomycin sulfate (100 U/ml) at 37 °C in a 5% CO_2 atmosphere. Cells were grown to 80% confluence, harvested from tissue culture flasks using 0.05% trypsin/0.02% EDTA and splitted in a ratio of approximately 1:10.

Stimulation by GM-CSF and inhibition by anti GM-CSF antibodies. For analysis of GM-CSF-induced modulation of IFN-γ-R gene activity, 80% confluent SW403 cells in 75 cm^2 tissue culture flasks were equilibrated in serum-free RPMI medium for 24 hours. Then, RPMI medium/0% FCS with 1,000 units/ml rhGM-CSF (Boehringer Mannheim, Germany) containing none or 2 μg/ml monoclonal mouse anti-human GM-CSF antibody (Genzyme, Boston, MA) was added. Cells incubated in serum-free medium only were used as control cells. After incubation for additional 24 hours, IFN-γ-R gene expression was determined.

Determination of GM-CSF, GMR and IFN-γ-R mRNA expression. RNA from SW403 cells was extracted as described by Kohrer and Domdey (7). cDNA synthesis from total RNA was achieved using the First Strand Synthesis Kit (Stratagene, Heidelberg, Germany) according to the manufacturer's recommendations. The first strand product was directly amplified, using the polymerase chain reaction as described by Perkin Elmer Cetus with the GeneAmp PCR Core Reagents Kit (Perkin Elmer Cetus, Norwalk, CT). PCR primers and the IFN-γ-R probe were generated by use of the "Primer" software package from the HUSAR data bank (DKFZ, Heidelberg, Germany). After electrophoresis of the amplificates on a 0.8% agarose gel, PCR samples were transferred subsequently to positively-charged nylon membranes by alkaline transfer according to the QIABRANE gravity-assisted transfer technique as described recently (8). After the transfer, the membrane was hybridized with the radioactive GM-CSF, (Clontech, Palo Alto, CA), GMR (Oncogene Science, Uniondale, NY) or IFN-γ-R oligonucleotide probe.

Immunoprecipitation and *in vitro* kinase assays. For determination of GM-CSF-induced activation of phosphotyrosine kinases, SW403 cells were incubated for 48 hours in RPMI 1640 serum-free medium to induce quiescence. The cells were then either left untreated or stimulated with GM-CSF (1,000 U/ml) for 20 minutes. Cultures were rinsed three times in ice-cold PBS containing 1 mM Na_3VO_4 and then lysed in 0.5 ml of phosphotyrosine lysis buffer according to Molloy et al (9). After centrifugation, supernatants were incubated with monoclonal mouse anti-human GMR (Cdw116) antibody (Genzyme) or monoclonal mouse anti-phosphotyrosine antibody (Zymed, San Francisco, CA) in the absence or presence of phosphotyrosine (0.2 mM). After addition of A/G PLUS-agarose conjugate and incubation at 4 °C overnight, GMR immune complexes were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose sheets. The GMR protein was detected using monoclonal mouse anti-human GMR α chain antibody (Santa Cruz Biotechnology) and the Western-Light Plus Chemiluminescent Detection system (Serva, Heidelberg, Germany). Anti-phosphotyrosine precipitates were washed with 50 mM HEPES (pH 7.4), 150 mM NaCl and resuspended in 50 μl kinase buffer (50 mM HEPES (pH 7.4), 10 mM $MnCl_2$, 0.5 μM ATP) containing 5 μCi -[^{32}P]ATP (Amersham Buchler, Braunschweig, Germany). Protein kinase reactions were carried out for 15 minutes at 37 °C. Reactions were stopped by addition of SDS-PAGE sample buffer and heating to 95 °C for 5 minutes, then labelled phosphotyrosine kinases were resolved by SDS-PAGE, blotted to nitrocellulose and visualized by autoradiography.

RESULTS

SW403 cells express GM-CSF and a functional GMR. By analysis with RT-PCR it could be demonstrated, that SW403 cells incubated in 10% FCS express significant amounts of GMR and GM-CSF mRNA, while under serum-free culture conditions only GMR transcripts were detectable. To check for the specifity of the amplification products, the resulting GMR and GM-CSF bands with a size of 682 and 140 base pairs, respectively, were blotted to nitrocellulose and hybridized with the corresponding antisense oligonucleotide probes, resulting in the identification as GMR and GM-CSF-specific gene fragments (Fig 1A, 1B). Further, the constitutive GMR expression in SW403 cells was demonstrated by immunoprecipitation of GMR with a GMR-specific mAb followed by Western blotting. In agreement with the results obtained with RT-PCR, the GMR of SW403 cells was synthesized both in serum-free medium and in medium containing 10% FCS (Fig. 1C).

To examine if in SW403 cells a GMR capable for signal transduction could be detected, the influence of GM-CSF on the activity of the interferon-γ receptor (IFN-γ-R) gene in the SW403 cell line

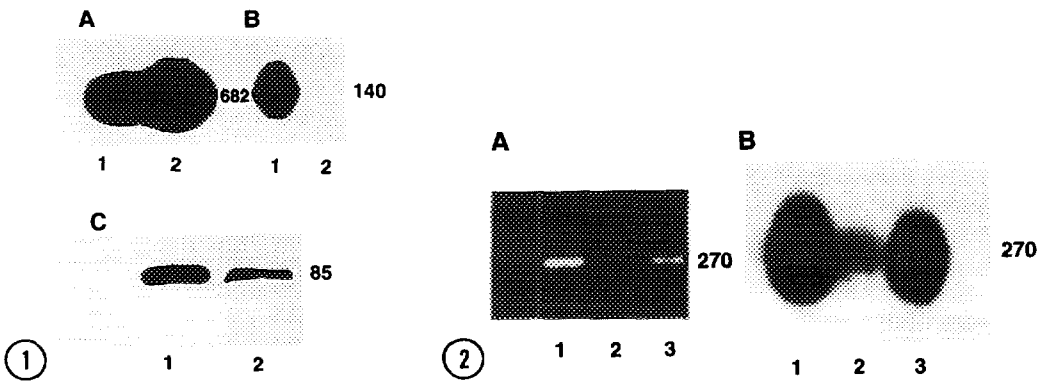

Fig.1. Demonstration of GMR and GM-CSF transcripts and of synthesis of GMR α-chain in SW403 cells. Random primers were used for reverse transcription of SW403 total RNA in cDNA. Following amplification by PCR with GMR (A) or GM-CSF (B) specific primer pairs and hybridization with gene specific probes, 682 base pairs GMR amplificates could be demonstrated in cells grown in serum-free medium (lane1) or in 10% FCS (lane 2) while 140 base pairs GM-CSF amplificates were only deteteable in cells grown in 10% FCS (lane 1), but not in 0% FCS (lane2). Autoradiograph exposure time was 14h at -80 °C. (C) Cells were grown to 70-80% confluence, washed, and then incubated in 10% FCS (lane 1) or serum-free medium (lane 2). After incubation for 24 hours, GMR α-chain of stimulated and unstimulated cells was immunoprecipitated by anti-human GMR α-chain monoclonal antibodies, separated on 7.5% polyacryamide gels and blotted. Precipitates containing the 85-kDa GMR α-chain were detected with a second monoclonal anti-human GMR α-chain antibody as described in the "Materials and Methods" section.

Fig.2. Effects of GM-CSF and anti GM-CSF mAbs on IFN-γ-R gene activity in SW403 cells. (A) 270 base pairs IFN-γ-R PCR products of RNA from SW403 cells that were incubated for 24 hours in serum free-medium (lane 1), in serum-free medium containing 1,000 U/ml rhGM-CSF (lane 2) or 1,000 U/ml rhGM-CSF and 2μg/ml anti rhGM-CSF mAb simultaneously (lane 3), followed by electrophoresis on a 1.2% agarose gel. (B) After blotting to nitrocellulose, amplificates were hybridized with an IFN-γ-R specifc antisense oligonucleotide. Autoradiograph exposure was 18h at -80 °C.

was investigated. Using RT-PCR with IFN-γ-R-specific primers, it could be demonstrated, that SW403 cells grown in serum-free medium express IFN-γ-R (Fig 2A). In addition, the amplified 899 bp sized gene fragment was hybridized with an IFN-γ-R-specific antinsense nucleotide to establish the identity with the IFN-γ-R gene (Fig. 2B). If SW403 cells growing in serum-free medium were stimulated with GM-CSF (1,000 units/ml), a significant reduction of the IFN-γ-R gene activity was detected. In contrast, the simultaneous incubation of SW403 cells with GM-CSF and a neutralizing anti-GM-CSF mAb (2 μg/ml) did not result in down-regulation of the IFN-γ-R gene expression. The incubation of SW403 cells with GM-CSF in a concentration range of 1.0-1,000 units/ml did not intensify DNA synthesis (data not shown).

The stimulation of SW403 cells with GM-CSF induces the activation of phosphotyrosine kinases. For the determination of protein kinase activity in SW403 cells after stimulation with GM-CSF, *in vitro* kinase assays with anti-P-TYR immunoprecipitates were carried out. As shown in Fig. 3, in the immunoprecipitates of unstimulated control cells several only slightly phosphorylated proteins ranging from 30 to 210 kDa could be demonstrated (lane 1). In comparison, anti-P-TYR immunoprecipitates of GM-CSF-stimulated cells exhibited a significantly more intense kinase activity. The most intense phosphorylation was detected in a 130 kDa band, other intensively phosphorylated proteins had molecular weights of 143 kDa and 210 kDa (lane 2). If the anti-P-TYR immunoprecipitation was carried out in the presence of excess phosphotyrosine, only a minimal phosphorylation of these bands could be observed (lane 3).

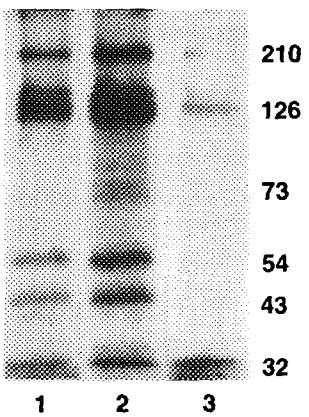

Fig.3. GM-CSF induced stimulation of protein kinases in SW403 cells. Anti P-TYR immunoprecipitates from serum-starved SW403 cells (lane 1) or from cells that had been stimulated for 20 min with GM-CSF (1,000 U/ml, lane 2) were assayed for in vitro kinase activity as described under "Materials and Methods". 0.2 μM phosphotyrosine was added as a control to the GM-CSF stimulated cell lysate (lane 3) prior to anti-P-TYR immunoprecipiation. ^{32}P-labeled proteins were subjected to SDS-PAGE, transferred to nitrocellulose and visualized by autoradiography. Exposure was 3 days at -80 C.

DISCUSSION

Only in a small number of cases the expression of GM-CSF and GMR has been described in human adenocarcinoma cell lines (6, 10, 11). Our resultes demonstrated, that SW403 cells synthesized GM-CSF after stimulation with 10% FCS and showed a constiutitive expression of a 85 kDa GMR, which is in agreement with the molecular weight of GMR in hematopoietic cells (12). Although the authentic GMR protein was expressed, it was not clear, if all components required for GM-CSF-induced signal transduction to the nucleus were present in SW403 cells. As it is known, that the incubation of human monocyte cell lines and peripheral blood monocytes with GM-CSF induces an IFN-γ-R down-regulation (13), the modulating effect of GM-CSF on the IFN-γ-R gene activity of SW403 cells was examined. While the IFN-γ-R was expressed intensively in serum-starved SW403 cells, the incubation of these cells with GM-CSF lead to an almost complete down-regulation of IFN-γ-R expression. This effect could not be observed in the presence of a neutralizing anti-GM-CSF mAb added simultaneously. In addition, a GM-CSF-mediated signal transduction up to the nucleus has been confirmed for SW403 cells. In agreement with a similar kinase activation pattern found in other cell lines, the results of the *in vitro* kinase assays demonstrated clearly the GM-CSF-dependent activation of a number of phosphotyrosine kinases (14, 15). Nevertheless, in contrast to findings for two other colon carcinoma cell lines (16), no role of GM-CSF as growth-stimulating factor could be observed in the SW403 cell line. The fact, that no growth stimulation is obtained, although GM-CSF-induced signal transduction is detected, could be explained with an activation of relevant signal paths below a critical limit, which is essential for the induction of DNA synthesis, or with the absence of a corresponding activating signal pathway. Our recent studies focus on a better understanding of the role of GM-CSF and GMR expression in SW403 cell lines, and on the characterization of GM-CSF-activated protein tyrosine kinases.

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