Journal of Cellular Biochemistry 80:625–634 (2001)

Expression of GM-CSF Receptors in Male Germ Cells and their Role in Signaling for Increased Glucose and Vitamin C Transport

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Abstract We studied the expression and function of the granulocyte-macrophage colony stimulating factor (GM-CSF) receptor in male germ cells. RT-PCR showed expression of mRNAs encoding the α- and β-subunits of the GM-CSF receptor in human testis, and the presence of the α- and β-proteins was confirmed by immunoblotting with anti-α and anti-β-antibodies. Immunolocalization studies showed the level of expression of GM-CSF α- and β-subunits in the germ line in the testis and in ejaculated spermatozoa. Receptor binding studies using radiolabeled GM-CSF revealed that bull spermatozoa have about 105 high-affinity sites with a K_d of 222 pM and ≈1100 low-affinity sites with a K_d of 10 nM. GM-CSF signaled, in a time- and dose-dependent manner, for an increased uptake of glucose and vitamin C. J. Cell. Biochem. 80:625–634, 2001. © 2001 Wiley-Liss, Inc.

Key words: GM-CSFr; sperm; vitamin C; 2-deoxy-D-glucose

GM-CSF is a pleiotropic cytokine that can stimulate proliferation, maturation and function of hematopoietic cells [Gasson, 1991]. In human host defense cells, GM-CSF stimulates increased glucose and vitamin C uptake probably mediated by changes in the functional activity of the facilitative hexose transporters (GLUTs) that permit the cellular uptake of these substrates by these cells [Vera et al., 1998]. GM-CSF mediates its effects via interaction with cell-surface receptors [Isfort and Ihle, 1990; Kanakura et al., 1990; Okuda et al., 1992; Eder et al., 1993; Hanazono et al., 1993; Ihle, 1995]. Receptors for GM-CSF are expressed on myeloid progenitors and mature mononuclear phagocytes, monocytes, eosinophils and neutrophils [Gasson et al., 1986; Park et al., 1986; Cannistra et al., 1990; Gasson, 1991]. The GM-CSF receptor is composed of two subunits, α and β [Hayashida et al., 1990; Kitamura et al., 1991]. The isolated α-subunit binds GM-CSF at low affinity (K_d: 1–7 nM). The isolated β-subunit does not bind GM-CSF by itself; however, in a complex with the α-subunit forms a high-affinity receptor (K_d: 20–250 pM).

Receptors for GM-CSF are also present in nonhematopoietic cells, such as placental trophoblasts, endothelial cells, oligodendrocytes in the central nervous system [Bussolino et al., 1989; Gearing et al., 1989; Baldwin et al., 1993; Brosnan et al., 1993] and some tumors [Spielholz et al., 1995; Rivas et al., 1998]. Although there is evidence indicating that GM-CSF may stimulate the growth of some nonhematopoietic tumor cell lines [Dedhar et al., 1988; Baldwin et al., 1989, 1991; Berdel et al., 1989; Jorasch-kewitz et al., 1990; Miyagawa et al., 1990; Nachbaur et al., 1990; Guillaume et al., 1993; Lang et al., 1994], the physiological role of the GM-CSF receptors expressed in normal or neoplastic nonhematopoietic tissue is unknown.

In these studies, we provide evidence that male germ cells express functional high-affinity

Grant sponsor: FONDECYT; Grant number: 199-0994.

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Received 5 July 2000; Accepted 8 September 2000

This article published online in Wiley InterScience, December XX, 2000.

GM-CSF receptors that signal an increased glucose and vitamin C uptake via spermatozoa functional facilitative hexose transporters recently described by us [Angulo et al., 1998]. These findings link hexoses and vitamin C uptake by spermatozoa to the action of colony-stimulating factors, providing evidence that humoral modulators of host defense stimulate increased transport of these substrates central to normal sperm physiology.

MATERIALS AND METHODS

Sample Collection

Human semen was collected in sterile plastic containers from healthy young men [Aitken and Clarkson, 1988]. Bull spermatozoa ejaculates were obtained from the Centro de Inseminación Artificial, Universidad Austral de Chile.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total human testis RNA was obtained from Clontech Laboratories, Inc. (Palo Alto, CA). Single-stranded cDNA synthesis and PCR were carried out as previously described [Heaney et al., 1995]. The primers used were: α-subunit primer: 5′AGCCCAGAGCAAAACACA, position 1009-1026 and 3′CCATGCCATTCCTACACCCT, position 1360-1379; β-subunit primer: 5′CTACAAGCCCAGCCCAGATGC, position 859-879 and 3′ACCCGTAGATGCCACAGAAGC, position 1390-1410. The PCR conditions were 94°C for 1 min and 65°C for 2 min for 35 cycles. PCR products were separated by 2% agarose gel electrophoresis and visualized by staining with ethidium bromide.

Binding Assays

For binding assays, 1×10^7 cells were suspended in phosphate-buffered saline (PBS), pH 7.4 containing 0.2% bovine serum albumin (BSA) and increasing concentrations of ^{125}I-labeled human GM-CSF (DuPont NEN) with or without excess unlabeled human recombinant GM-CSF (R&D). After incubation for 15 min at 4°C, the cells were centrifuged for 5 min at 4°C through a cushion of fetal bovine serum and the cell pellets were washed with cold PBS. Bound GM-CSF was quantitated by γ-spectrometry.

Uptake Assays

Uptake assays in spermatozoa were measured as described previously [Angulo et al., 1998]. When appropriate, recombinant human GM-CSF was preincubated with sperm cells as indicated in the figure legends.

Immunoblotting

Spermatozoa membrane proteins were obtained as previously described [Angulo et al., 1998] and resolved by SDS-PAGE (30 μg per lane) in a 10% polyacrylamide gel and transferred to immobilon (Millipore, Bedford, MA). The antibody blots were developed by chemiluminescence (Amersham Corporation, Arlington Heights, IL).

Immunocytochemistry

For immunoperoxidase localization, spermatozoa were spread onto slides, fixed in buffered paraformaldehyde-acetone, treated with 0.3% H_2O_2 for 5 min and incubated for 60 min at room temperature in 5% BSA-PBS pH 7.4, followed by incubation overnight at 4°C with anti-α or anti-β GM-CSF receptor subunit antibodies (1:500) (Sta. Cruz Biotechnology, Inc.) in 1% BSA-PBS pH 7.4 and 0.3% Triton X-100. Cells were washed and incubated with anti-rabbit IgG-horseradish peroxidase (1:100, Amersham) for 2.5 h at room temperature. Immunostaining was developed using 0.05% diaminobenzidine and 0.03% H_2O_2. As controls, cells were incubated with antibodies preabsorbed with the respective peptide used to generate the antibodies. Cells were counterstained with hematoxylin. Expression of GM-CSF receptors in human testis sections was similarly determined.

In Situ Hybridization

mRNA localization in testis sections was performed as previously described [Baldino and Lewis, 1989; Concha et al., 1993]. Thin sections prepared from archived paraffin-embedded tissue blocks were used. The antisense oligoprobes described above for RT-PCR assays were labeled at the 3′ end with terminal transferase and digoxigenin-11-dUTP (Boehringer-Mannheim, Germany) hybridized and visualized by 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolphosphate using anti-digoxigenin Fab fragments coupled to alkaline phosphatase. As controls, sections were treated with RNase.

RESULTS

GM-CSF Receptors are Expressed in Human Germ Cells

A band of ≈370 nucleotides, the expected size of the amplification product for the α-subunit mRNA, was amplified by RT-PCR from human testis RNA (Fig. 1). A similar approach using primers complementary to the β-subunit of the GM-CSF receptor revealed the expected amplification product of ≈570 nucleotides (Fig. 1). The size of the amplified bands corresponded exactly to the size of the respective bands amplified from RNA obtained from HL-60 cells which express abundant mRNA for the α- and β-subunits of the GM-CSF receptor (data not shown). In additional controls, we observed no amplification products in reactions in which the reverse transcriptase was omitted, confirming the absence of contaminating DNA in the RNA preparation. Expression of mRNAs for both GM-CSF receptor subunits was further analyzed by in situ hybridization using digoxigenin-labeled probes specific for the α- and β-subunits (Fig. 1). Strong signals were obtained in nuclei and nuclear periphery in germ cells, from spermatogonia to spermatids. The most strongly labeled cells corresponded to spermatocytes. All cells labeled with the α-probe were also positive for the β-probe, indicating that they express both subunits simultaneously. There was no apparent difference in the intensity of labeling of the different cells with the α- or the β-probe, suggesting the presence of equivalent levels of both mRNAs in the cells. Control experiments using RNase treated sections revealed no labeling of the cells, confirming the specificity of the reaction with the α- and β-antisense probe (Fig. 1B and D).

The presence of the α- and β-subunits of the GM-CSF receptor in adult human testis was confirmed by immunolocalization with antibodies specific for each subunit (Fig. 2A and B). The immunoreactivity was associated with the plasma membrane and was also observed intracellularly in all germ cells. The intensity of the immunolabeling with the anti-α-antibody was consistently higher than that of the anti-β-antibody. The most strongly labeled cells with either antibody corresponded to spermatocytes, similar to the results observed for the GM-CSFr subunits mRNA localization (Fig. 1). The immunoreactivity decreased at increasing stages of maturation and differentiation. Control sections incubated with antibodies preadsorbed with the corresponding peptides for the α- and β-subunits of the receptor did not show a positive signal (Fig. 2C and D).

Ejaculated human spermatozoa were immunoreactive with both antibodies, with intense immunostaining observed along the tail (Fig. 3A and B). The strongest signal was observed in the mid piece of the tail, with the signal elicited with the anti-α-antibody being consistently stronger than that of the anti-β-antibody. No immunoreactivity was observed when preabsorbed antibodies were used (data not shown). Immunoblotting of membrane proteins extracted from human spermatozoa revealed protein bands reacting with both antibodies. The α-subunit antibody reacted with a main protein band that migrated with an apparent M_r of 80–82 kDa, and a less intensively stained band with an apparent M_r of 45 kDa. The β-subunit antibody reacted with a unique protein band with apparent M_r of 105 kDa (Fig. 3E).

Bull Spermatozoa Express Functional GM-CSF Receptor

The RT-PCR, in situ hybridization, immunolocalization and immunoblotting data are consistent with the expression of the α- and β-subunits of the GM-CSF receptor in human germ cells, and it is therefore reasonable to assume that these receptors are functional and able to transduce a signal. Although it is possible to use human ejaculated spermatozoa for functional studies, the type of analysis necessary to study the functional properties of the GM-CSF receptors requires obtaining a highly purified and homogeneous population of human spermatozoa, which is very difficult. Because of this, we looked for alternative models to analyze the issue of the functional status of the spermatozoa GM-CSF receptors. In exploratory studies we obtained data indicating the presence, in bull spermatozoa, of proteins reactive with anti-human α- and β-subunit antibodies. Detailed immunohistochemical analysis indicated that bull spermatozoa were immunoreactive with both α- and β-antibodies, with the immunoreactivity specifically associated to the sperm tail (Fig. 3C and D). Similar to human spermatozoa, there was always a greater intensity of staining with the α-antibody as compared with the β-antibody in bull spermatozoa. Confirming the results of the immunolocalization studies, immunoblotting of membrane

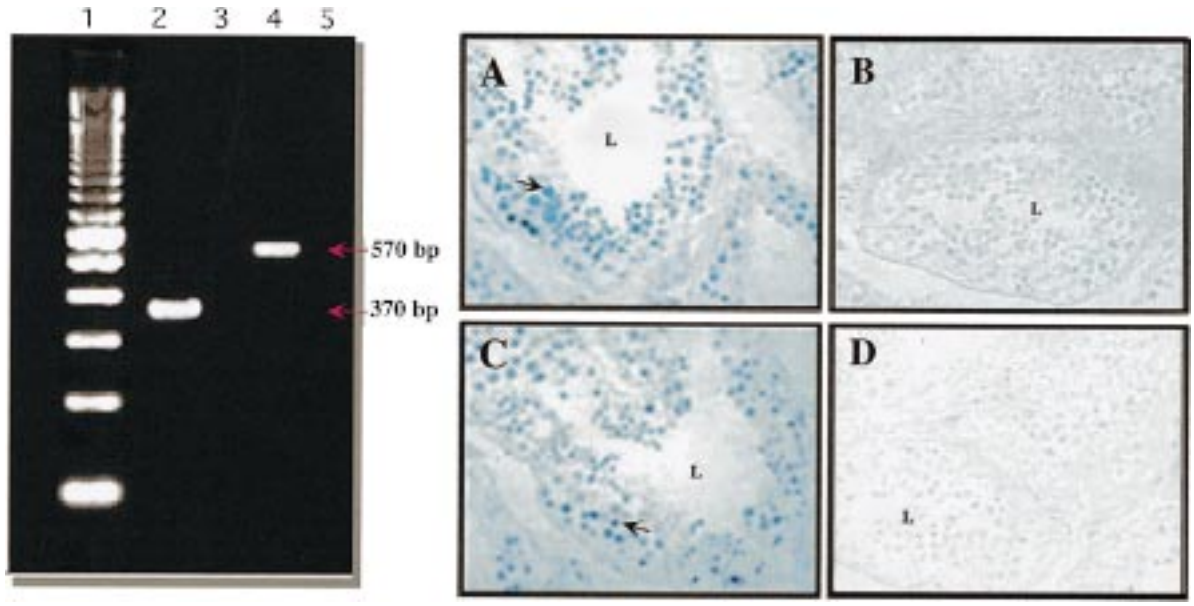

Fig. 1. Expression of GM-CSF receptors in human testis. Left panel: RT-PCR analysis. Total testis RNA was subjected to RT-PCR using primers specific for the α- or β-subunits of the GM-CSF receptor. PCR products were size-fractionated on 2% agarose gels and visualized by staining with ethidium bromide. The amplification products corresponding to the α (370 bp) and the β (570 bp) subunits of the GM-CSF receptor is shown in lanes 2 and 4. A reaction in which the cDNA synthesis step was performed in the absence of reverse transcriptase was used to control for the presence of contaminant DNA in the RNA samples (lanes 3 and 5). The migration in the agarose gel of a series of DNA 100-mer size standards is shown in lane 1. Right panel: In situ hybridization in human testis. Expression of mRNA GM-CSF receptor is seen on human testis sections. **A:** Corresponds to α-subunit and **C:** corresponds to the β-subunit. **B** and **D:** Correspond to the respective negative controls using RNase treated sections. The arrows show positive staining in nuclei and nuclear periphery (original magnification, 90 ×). L: Lumen of the seminiferous tubule.

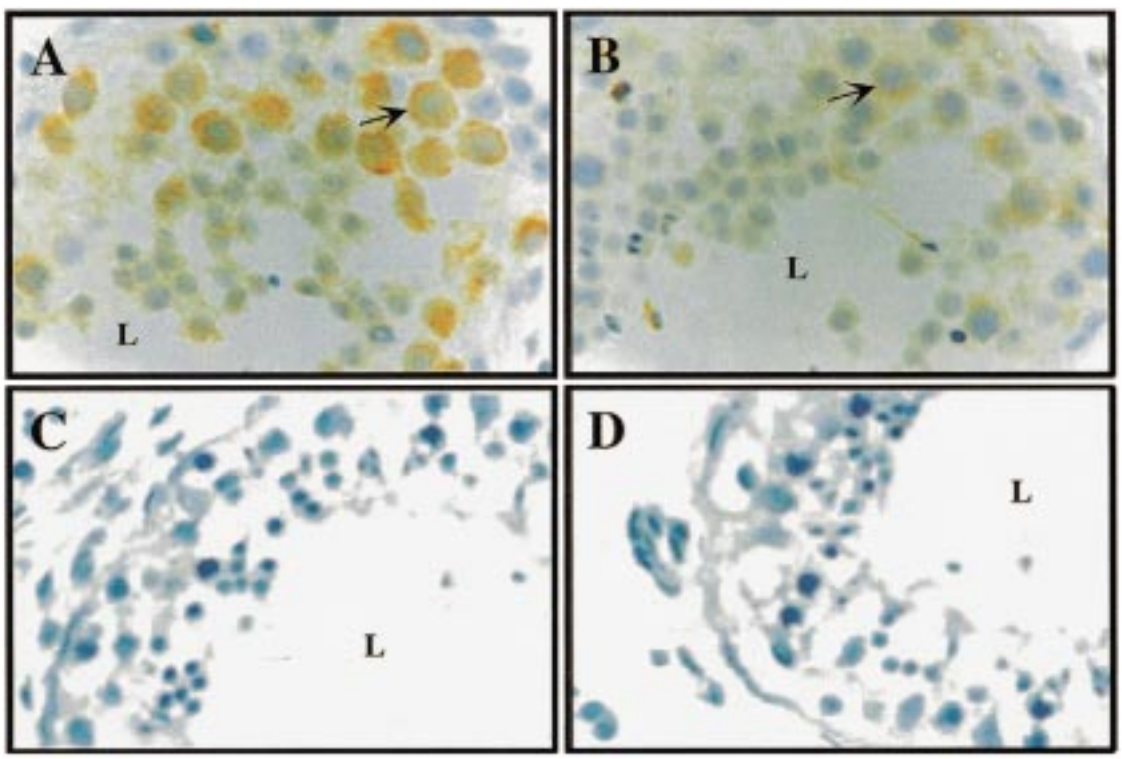

Fig. 2. A, B: Immunostaining with anti-human GM-CSF receptor antibodies in human testis. Testis sections were incubated with anti-α (**A** and **C**) or anti-β subunit (**B** and **D**) antibodies in the absence (A and B) or the presence (C and D) of the peptides used to elicit them followed by incubation with a secondary antibody conjugated to horseradish peroxidase. The arrows show positive staining at the membrane and perinuclear level (A and B) (original magnification, 160 ×). L: Lumen of the seminiferous tubule.

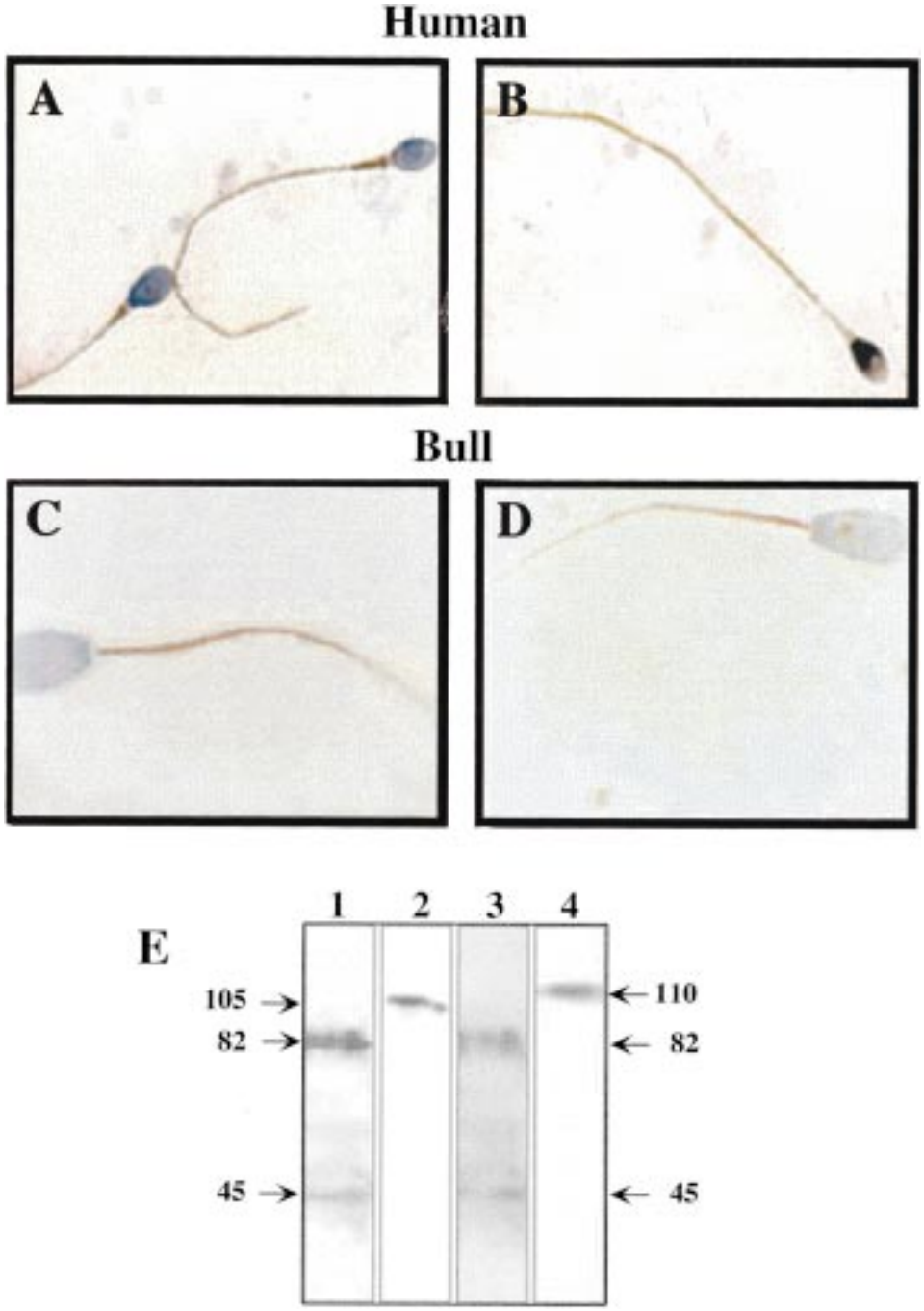

Fig. 3. Immunolocalization of GM-CSF receptors in human and bull spermatozoa. Human (**A** and **B**) and bull (**C** and **D**) spermatozoa were spread onto coated slides and probed with the anti-α (A and C) and anti-β (B and D) subunit antibodies, followed by incubation with a secondary antibody conjugated to horseradish peroxidase (original magnification 900 ×). Lower panel **E:** Identification of GM-CSF receptor in human and bull spermatozoa by immunoblotting. Membrane proteins isolated from human (lanes 1 and 2) and bull (lanes 3 and 4) spematozoa were fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate, transferred to nitrocellulose membranes, and probed with anti-α (lanes 1 and 3) and anti-β (lanes 2 and 4) subunit antibodies, followed by incubation with a secondary antibody coupled to peroxidase. ECL: Sizes on the left and the right are kDa and indicate the migration of molecular weight standards.

proteins extracted from bull spermatozoa demonstrated the presence of protein bands reacting with both antibodies. The anti-α-antibody reacted with a protein band with an apparent M_r of 80–82 kDa, although a less intense band with an apparent M_r of 45 kDa was also evident (Fig. 3E). On the other hand, the anti-β-antibody reacted with a unique band with an apparent M_r of 110 kDa (Fig. 3E).

Binding studies revealed that human GM-CSF bound to bull spermatozoa cells in a dose-dependent and saturable manner (Fig. 4A). Scatchard analysis of the binding data (Fig. 4B) revealed that the bull spermatozoa expressed ≈105 high-affinity binding sites for GM-CSF with a K_d of 222 pM, and 1100 low-affinity binding sites with a K_d of 10 nM. Thus, the binding data confirmed the presence of high- and low-affinity GM-CSF receptors in bovine spermatozoa, which is consistent with the immunochemical data indicating the presence of the α- and β-subunit of the GM-CSF receptor in these cells.

To test the function of the GM-CSF receptor in sperm cells, we examined changes in glucose and vitamin C uptake in reponse to GM-CSF treatment. Bull spermatozoa incubated in the presence of different concentrations of GM-CSF showed increased uptake of deoxyglucose and dehydroascorbic acid, the oxidized form of vitamin C (Fig. 5A and B). GM-CSF induced a dose-dependent increase in deoxyglucose uptake, with half-maximal activation observed at 100 pM GM-CSF. Maximal activation was observed at 10 nM GM-CSF with a 2.8-fold increase in deoxyglucose uptake.

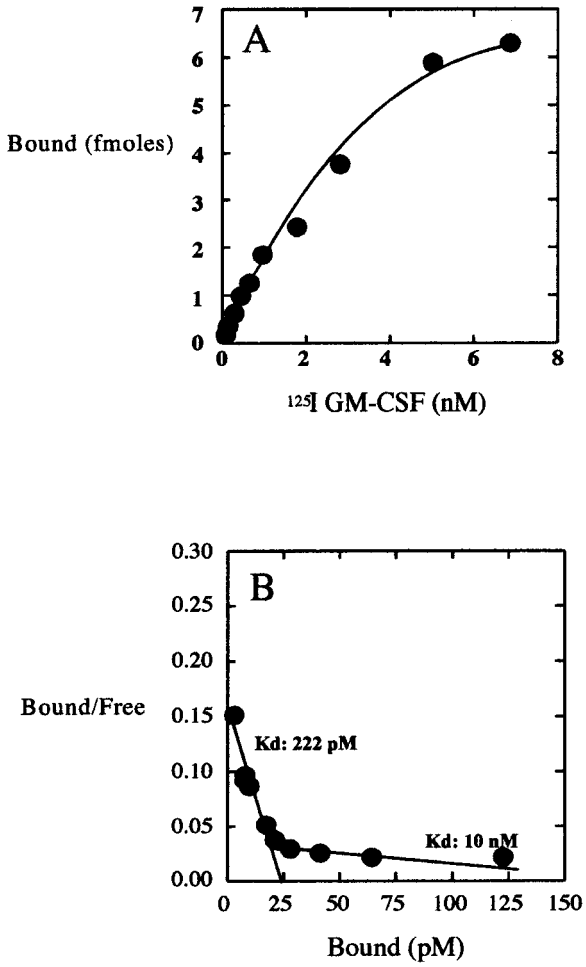

Fig. 4. **A:** Binding analysis. Bull spermatozoa were incubated with radiolabeled GM-CSF at concentrations that ranged from 5 pM to 7 nM. GM-CSF binding was dose dependent and saturable approximately at 7 nM. **B:** Scatchard analysis of data from B showing the presence of two classes of binding sites in the sperm cells.

DISCUSSION

The physiologic role of GM-CSF receptors in nonhematopoietic tissue is unknown. We report here a detailed study addressing the issue of GM-CSF receptor expression in male germ cells. The presence of GM-CSF receptors in male germ cells was defined by RT-PCR, in situ hybridization, immunolocalization, ligand binding and functional assays. RT-PCR showed that human germ cells express mRNAs for the α- and β-subunits of the GM-CSF receptor, and the immunolocalization experiments confirmed the presence of the α- and β-proteins in all germ cells, from spermatogonia to spermatids.

The results of the immunoblotting experiments, using anti-α and anti-β-antibodies, support the notion that the human spermatozoa GM-CSF receptor has a subunit structure similar to the GM-CSF receptor present in hematopoietic cells for which the α- and β-subunits have been characterized in detail.

Although the cDNA for the α-subunit encodes a protein with a predicted M_r of 44 kDa, analysis of the mature α-subunit by electrophoresis in polyacrylamide gels revealed an heterogeneous protein band with an estimated M_r of 75–85 kDa due to *N*-glycosylation at several of the predicted 11 *N*-glycosylation sites [Gearing et al., 1989]. In a similar fashion, although the cDNA for the β-subunit encodes a protein with a predicted M_r of 110 kDa, the mature β-subunit migrates in polyacrylamide

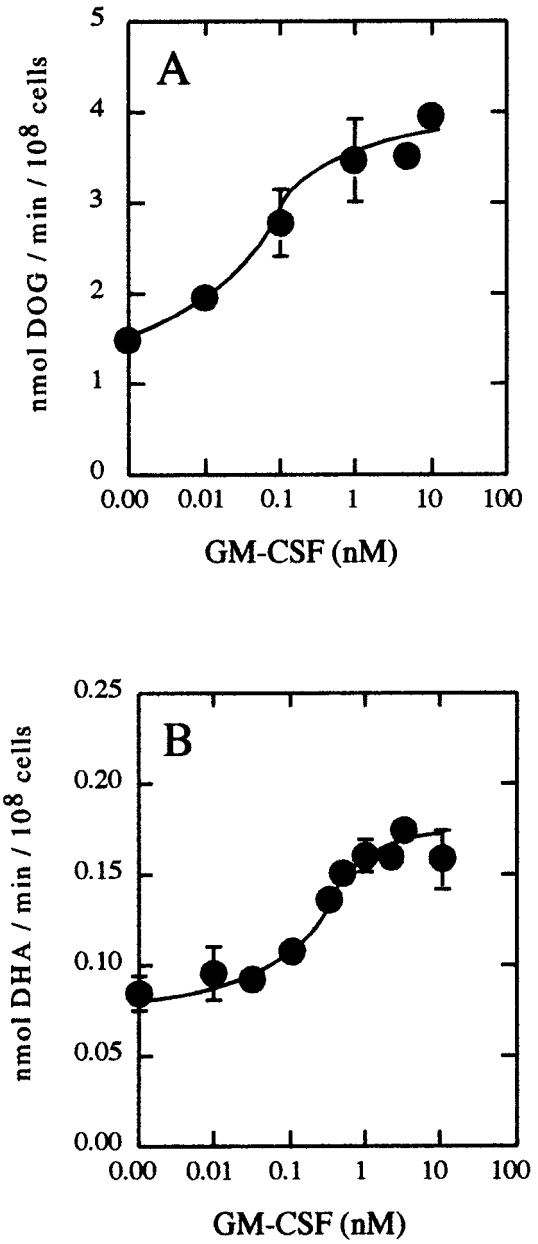

Fig. 5. Effect of GM-CSF concentration on DOG and DHA uptake in bull spermatozoa. The uptake of DOG (**A**) and DHA (**B**) in bull spermatozoa in response to concentrations of GM-CSF ranging from 0 to 10 nM at 30 min treatment time is shown. The results are displayed as nmol/min in DOG, DHA uptake and in cells treated with GM-CSF relative to cells that were not treated.

as an heterogeneous protein band with an estimated M_r of 120 kDa.

In human spermatozoa, the anti-α-antibody reacted with protein bands of 82 and 45 kDa, which we propose correspond to the fully glycosylated and the unglycosylated α-subunit, respectively. In a similar manner, the 105 kDa band recognized by the anti-β-antibodies in human spermatozoa may represent an unglycosylated β-subunit.

A central finding of this work is that bull spermatozoa appear to express the bovine homolog of the human GM-CSF receptor. This finding is supported by structural as well as functional data. First, antibodies directed against the α- and β-subunits of the human GM-CSF receptor reacted strongly and specifically with protein bands present in bull spermatozoa. Moreover, the immunoblotting data suggest that the subunit structure of this putative bovine GM-CSF receptor is similar to that of the human receptor. The anti-α-antibody reacted with protein bands of 82 and 45 kDa present in total membrane preparations of bull spermatozoa, which by homology with the human situation we propose may correspond to the fully glycosylated and the unglycosylated α-subunit of the bovine GM-CSF receptor, respectively. In a similar manner, the 110 kDa band recognized by the anti-β-antibodies in bull spermatozoa may represent the bovine β-subunit. This is of interest because the only other GM-CSF receptor for which there is structural information is the murine receptor, and the subunit structure of this receptor is clearly different from the human one. Moreover, antibodies specific for the different subunits of the human GM-CSF receptor fail to react with their mouse counterparts. The opposite is also true, antibodies against the mouse GM-CSF receptor do not cross-react with the human receptor α- or β-subunits.

Since the antibodies used in these experiments are antipeptide antibodies and they were used after fractionation of the bull sperm membrane proteins by denaturing electrophoresis, the immunoblotting data support the concept that the α- and β-subunits of the bovine GM-CSF receptors share sequence homology with the human subunits. In support of this concept is the data, from binding studies using radiolabeled human GM-CSF, showing the existence of specific GM-CSF binding sites in bull spermatozoa. Because of the antibody data, and in homology with the interpretation of similar experiments using human cells, we defined these GM-CSF binding sites as representing bona fide GM-CSF receptors. Accordingly, the results of the ligand-binding studies can be interpreted as indicating that bull spermatozoa express both high- and low-affinity GM-CSF

receptors. This is a phenomenon that has been previously reported for human cells and corresponds to a molar excess of α as compared to β-subunit at the protein level. In fact, the number of high-affinity receptors present in bull spermatozoa was similar to the number present in cells of hematopoietic origin in which GM-CSF induces proliferation and differentiation [Chiba et al., 1990], and in nonhematopoietic cells such as mouse fibroblasts expressing the human high-affinity receptors which respond to GM-CSF with cell proliferation and protein phosphorylation [Eder et al., 1993; Watanabe et al., 1993a; Watanabe et al., 1993b]. The identification of 1,100 low-affinity and 105 high-affinity GM-CSF binding sites in bull spermatozoa can be interpreted as indicating the presence of an excess of α as compared to β-subunit in these cells.

Biological response analyses confirmed the presence of functionally active GM-CSF receptors, responsive to human GM-CSF, in bull spermatozoa. Human GM-CSF was able to induce increased uptake of deoxyglucose in a dose-dependent manner that closely matched the effect of this cytokine in human hematopoietic cells. The effect of GM-CSF on deoxyglucose uptake was clearly evident at low concentrations of GM-CSF, around 100 pM, which is consistent with high-affinity K_d detected in the binding experiments with radiolabeled human GM-CSF. Deoxyglucose uptake increased further at concentrations of GM-CSF in the nanomolar range, which is consistent with the low-affinity K_d observed in the binding studies. In human cells, the myeloid colony-stimulating factors stimulate increased glucose uptake in target cells, presumably to provide increased metabolic fuel for heightened cellular activity. In the case of GM-CSF, studies with cells expressing the isolated α-subunit as well as the α/β-complex indicated that signaling for increased glucose uptake is mediated through the GM-CSF receptor α-subunit [Spielholz et al., 1995; Nicola 1997]. In addition to increasing the cellular uptake of glucose, the colony-stimulating factors also increase the cellular uptake of vitamin C in neutrophils and monocytes, an effect that appears to be mediated through a direct activation of the facilitative hexose transporters [Vera et al., 1998].

This study reports increased glucose and oxidized vitamin C uptake by bull spermatozoa stimulated with human GM-CSF. Although the action of hematopoietic growth factors and their receptors in normal testicular physiology is unknown, our results suggest that GM-CSF may have a functional role in the normal physiology of the male germ cell. Increased glucose uptake may be related to an increased use of sugars as metabolic fuels. Human and bull spermatozoa express several members of the family of facilitative hexose transporters and efficiently transport the energy-producing sugar glucose and fructose [Angulo et al., 1998]. Moreover, the glucose transporters are efficient transporters of the oxidized form of vitamin C, dehydroascorbic acid [Vera et al., 1993, 1995; Angulo et al., 1998]. Humans cannot synthesize vitamin C, and therefore the vitamin must be provided in the diet and transported intracellularly [Rose, 1988]. Vitamin C is fundamental to human physiology and appears to be essential for the normal development of the male germ cells [Chinoy et al., 1986]. Low concentrations of vitamin C are associated with the production of poor quality sperm and decreased fecundating capacity [Dawson et al., 1987, 1992]. It is feasible that an increased uptake of vitamin C may be related to increased anti-oxidant defense in a cell such as the spermatozoa that is capable of generating highly oxidative species in a manner in many ways similar to the generation of superoxide anions by host defense cells.

In brief, we demonstrated the presence of both subunits of the GM-CSF receptor in human and in bovine male germ cells, and show that bull spermatozoa express functional GM-CSF receptors that signal for increased glucose and vitamin C uptake. These results establish that GM-CSF, a typical hematopoietic growth factor, may have an important role in the physiology of male germ cells.

ACKNOWLEDGMENT

The authors thank David W. Golde from the Memorial Sloan-Kettering Cancer Center for their continuous interest and support.

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