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Mast cells can revert dexamethasone-mediated down-regulation of stem cell factor

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Abstract

Mast cell hyperplasia can be causally related with chronic inflammation and liver fibrosis. Their survival and proliferation is dependent upon locally produced growth factors, the major one being the stem cell factor (SCF). Glucocorticoids can decrease mastocytosis, down-regulating the mast cell production of pro-inflammatory factors or inhibiting the expression of SCF in stroma. We compared dexamethasone effect on SCF expression in co-cultures of mast cells with NIH/3T3 fibroblasts or with primary cultures of activated hepatic stellate cells. Dexamethasone abrogated the NIH/3T3 stroma capacity to sustain mast cell proliferation, but not of hepatic stellate cells, at the post-transcriptional level. Mast cells reverted completely dexamethasone effect on hepatic stellate cells, increasing their SCF synthesis and transport. In both models, dexamethasone inhibited the mast cell spreading on the stroma, which was thus not required for mast cell survival and proliferation. Liver pathologies associated with mast cell hyperplasia are not expected to be sensitive to glucocorticoid treatments. © 2001 Published by Elsevier Science B.V.

Keywords: Inflammation; Fibrosis; Mast cells; Proliferation; Stem cell factor; Glucocorticoids

1. Introduction

Mast cells are produced in the bone marrow, from which they exit as undifferentiated precursors, migrate through the blood vessels, and settle in tissues where they differentiate into various subsets of resident mast cells. These processes are directly controlled by the tissue microenvironment as well as by growth factors and cytokines such as stem cell factor (SCF), interleukin-3, interleukin-9, interleukin-10 (Li and Krilis, 1999). Simultaneously, mast cells produce a vast array of mediators that can activate the adjacent cells, participate in paracrine controls of the tissue homeostasis, and trigger or intensify the tissue inflammatory processes. Several pathologies are associated with a mast cells hyperplasia such as allergic reactions, asthma, inflammation, fibrosis, and response to intestinal parasite infections. In liver, a co-distribution of mast cells with chronic inflammatory reactions and fibrosis has been reported (Farrell et al., 1995; Armbrust et al., 1997; Tsuneyama et al., 1999), but a causal relationship between the mast cell hyperplasia and liver fibrosis is still debated (Okazaki et al., 1998).

Most mast cell-related pathologies are treated through administration of glucocorticoids, which down-regulate or suppress the mast cell production of cytokines and inflammatory mediators (Williams and Coleman, 1995; Eklund et al., 1997; Fushimi et al., 1998). In addition, glucocorticoids were reported to reduce the number of resident tissue mast cells in skin, bronchial mucosa, and intestine (Lavker

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and Schechter, 1985; Pipkorn et al., 1989; Jeffery et al., 1992). The capacity of glucocorticoids to decrease the mast cell numbers is due, at least in part, to their control of the expression of SCF, which is the main growth factor for human and rodent mast cells both in vivo and in vitro. However, recent studies have given conflicting results on this issue. SCF expression was shown to be down-regulated by glucocorticoids in human and murine skin fibroblast both at the mRNA and protein level (Finotto et al., 1997), but it was either up- or down-regulated in human lung fibroblasts depending on the duration of treatment (Kassel et al., 1998). In human bone marrow fibroblasts, glucocorticoids were reported to increase the SCF expression, while no effect was detected in bone marrow mesenchymal cells (Lindenberg et al., 1995; Thalmeier et al., 1996; Haynesworth et al., 1996).

In liver, SCF is produced by biliary epithelial cells in various disorders associated with peribiliary fibrosis, but not by normal biliar cells (Tsuneyama et al., 1999). It is also expressed in hepatic stellate cells during liver regeneration (Fujio et al., 1994). In a previous in vitro study, we have shown that murine hepatic stellate cells, derived from fibro-granulomatous reactions to *Schistosoma mansoni* eggs, could be induced to express SCF by mast cells, and could support in co-culture both murine and human mast cell proliferation (Brito and Borojevic, 1997). Similar results were obtained in a recent study on human and rat hepatic stellate cells, in which an increase of SCF mRNA and protein was described, as well as an enhanced production of SCF in fibrotic liver tissue (Gaça et al., 1999).

In the present work, we have addressed the question of the glucocorticoid-mediated regulation of SCF expression by hepatic stellate cells, and its effect on their capacity to sustain the survival and proliferation of murine and human mast cells. Our results show that mast cells could revert the inhibitory effect of dexamethasone on SCF in hepatic stellate cells, but not in NIH/3T3 cells. These results indicate that the response of stroma and mast cells to intercellular mediators and dexamethasone are specific for different cell types.

2. Materials and methods

2.1. Materials

Reagents and sources are as follows: Dulbecco's minimum essential medium (D-MEM), RPMI 1640 medium, collagenase (type I), hyaluronidase (type I), toluidine blue, trypan blue, streptomycin, penicillin, HEPES and trypsin were obtained from Sigma, St. Louis, MO, USA. Fetal bovine serum (FBS) was from Cultilab, Campinas, SP, Brazil. Percoll was from Pharmacia, Uppsala, Sweden. Plastic tissue culture dishes were obtained from Nunc, Roskilde, Denmark. Oligo (dT) 12–18 primer, dATP, dCTP, dGTP, dTTP, SuperScript II Reverse Transcriptase, dithiotreitol, *Taq* DNA polymerase, TRIzol® Reagent and 100 bp DNA ladder were obtained from GIBCO BRL, Gaithersburg, MD, USA. Dexamethasone (Decadron) was from PRODOME, Campinas, SP, Brazil.

2.2. Cells and cell cultures

NIH/3T3 cells were obtained from the Rio de Janeiro Cell Bank (PABCAM, Federal University, Rio de Janeiro, RJ, Brazil). When not stated otherwise, cells were maintained in the "standard culture medium" (RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 g/l $NaHCO_3$, 2 g/l HEPES buffer pH 7.4), at 37°C, under 5% CO_2.

Activated hepatic stellate cells were isolated from granulomas elicited in mouse livers by schistosomal infection, as previously described (Alvarez-Silva et al., 1993). Briefly, C3H/HeN mice were infected by 40 cercariae of *S. mansoni* and sacrificed 90 days after infection. Granulomas were isolated from the homogenized liver tissue by sedimentation, and digested by collagenase. The harvested cells were seeded into tissue culture flasks and subcultured by trypsinization, during which the trypsin-resistant macrophages were eliminated. After the third passage, a homogeneous connective tissue cell cultures were obtained, termed GR cells. These cells were fully described and characterized in previous studies (Boloukhère et al., 1993).

Dexamethasone effect on interaction of liver connective tissue stromas with mast cells was compared to that of the NIH/3T3 cells, which were chosen due to their capacity to sustain murine mast cells in vitro (Levi-Schaffer et al., 1986). Murine peritoneal mast cells were isolated and purified from peritoneal cavities of C57BL/10J mice as previously described (Kobayashi et al., 1986). Briefly, sterile balanced salt solution (BSS) containing 0.1% (w/v) gelatin and 5 U/ml heparin was injected into the peritoneal cavity, and subsequently aspirated from each animal. Cells were pooled and layered over Percoll density-gradient and centrifuged. Approximately $4–6 \times 10^4$ mast cells (93% purity) were obtained per animal. Mast cells were identified using the toluidine blue metachromatic staining.

Co-culture of murine mast cells and GR cells were prepared as previously described for co-cultures of rat peritoneal mast cells with NIH/3T3 cells (Levi-Schaffer et al., 1985). Briefly, GR cells were seeded into 24-well culture plates at a concentration of 1.1×10^4 cells/well in 500 μl standard culture medium. The culture medium was changed every 2 days until the GR cells reached confluence. Subsequently, 1.1×10^4 purified murine mast cells, suspended in 500 μl standard medium, were seeded onto the GR cells, and the culture medium was replaced every 48 h. The number of mast cells was monitored at days 3, 6 and 9 of culture. They were fixed with 4% formaldehyde, stained with toluidine blue and the total number of

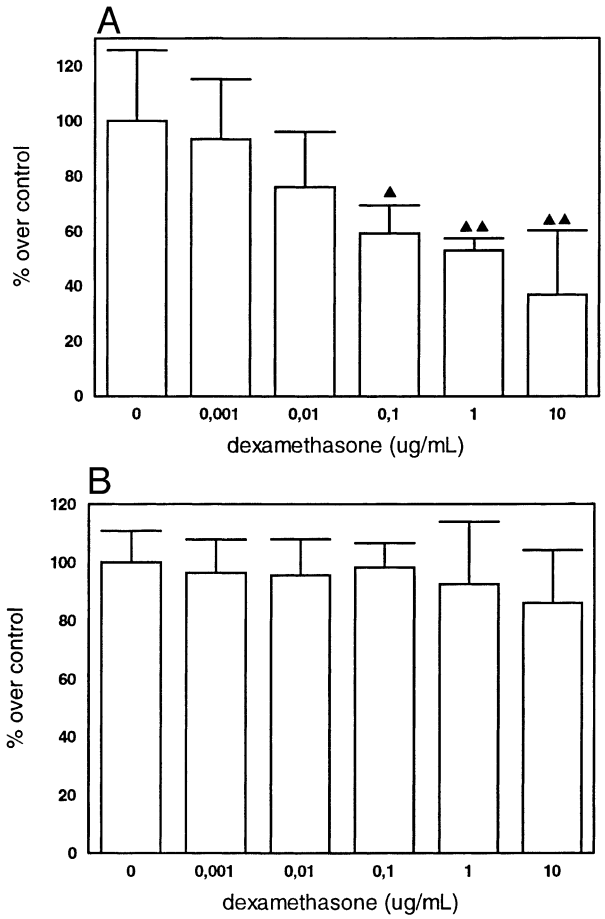

Fig. 1. Dose–response effect of dexamethasone on murine mast cells (MMC) maintained in co-culture with GR cells and 3T3 cells. (A) murine mast cells co-cultured with NIH/3T3 cells for 6 days with increasing concentrations of dexamethasone (0.001–10 μg/ml). Low concentration of dexamethasone (0.01 μg/ml) was able to decrease the relative number of mast cells to 76±20%, and increasing doses of dexamethasone decreased their relative numbers to 59.3±10.1% (▲ $P < 0.05$ compared to control), 53±4.4% and 36.8±23.4% (▲▲ $P < 0.01$). (B) Co-cultures of murine mast cells with GR cells treated as above, showed a slight but not significant decrease of mast cells numbers at 10 μg/ml. Results represent the relative mast cells numbers (mean±SE) of two experiments done in quadruplicate.

metachromatic cells was counted under a microscope equipped with an eyepiece containing a measuring grid.

2.3. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR)

RNA was extracted from 0.5 to 1×10^6 cells using TRIzol® Reagent following the standard protocols. The total RNA was dissolved in water, quantified and stored at −20°C. The total RNA (5 μg) was reverse-transcribed into cDNA, and the PCR was performed as previously described (Ehlers and Smith, 1991). B-actin primer sequences were: 5′GTG GGC CGC TCT AGG CAC CAA 3′, and 5′CTC TTT GAT GTC ACG CAC GAT TTC 3′, which amplify a 540-bp fragment (Alonso et al., 1986). SCF primer sequences were 5′CCG GAT CCT GGA GCT CCA GAA CAG CTA A3′ and 5′GGC TGC AGT CCA CAA TTA CAC CTC TTG AA3′, amplifying the major 910-bp fragment and occasionally a smaller 830-bp fragment (Flanagan et al., 1991). Routinely, RT-PCR was done using 30 rounds of amplification. The products were separated in 2% agarose, stained with ethidium bromide and visualized by ImageMaster® VDS (Pharmacia Biotech).

The semi-quantitative RT-PCR was initially done in 15, 20, 25 and 30 cycles, in order to define the range of linear increase of amplification. The final reaction mixture was divided in two tubes (500 μl) and 5 μl of β-actin or SCF primers were added to each one. The products from the 25th cycle were compared by densitometry (Imaging Densitometer GS-690, Bio-Rad, Hercules, CA, USA) using the

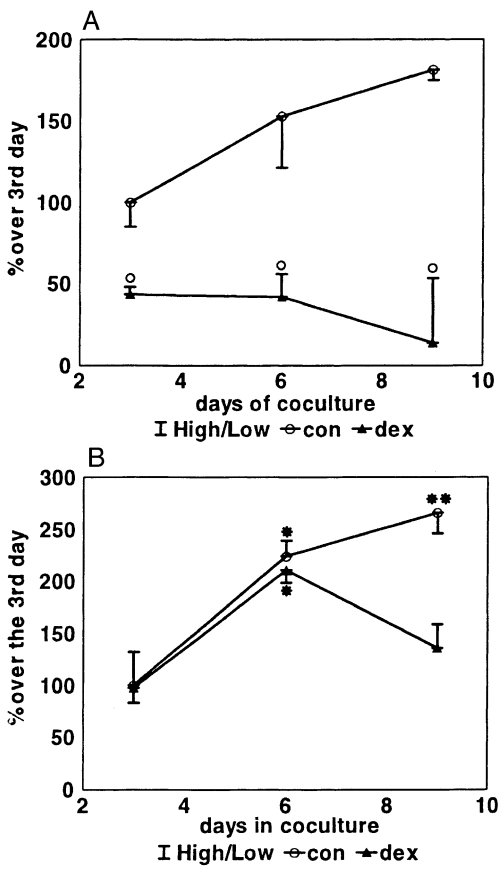

Fig. 2. Co-culture of murine mast cells (MMC) with NIH/3T3 or GR cells treated with dexamethasone (10 μg/ml) for 3, 6 and 9 days. (A) murine mast cells/3T3 showed a reduction of the relative number of mast cells to 43.4±4.4% at third day (○ $P < 0.01$ compared to control at the same day) and to 42±4.4% and 13.8±39.5% after at days 6 and 9, respectively (○ $P < 0.01$). (B) At 6 days, in cultures of murine mast cells over GR cells with dexamethasone, mast cells increased their number to 210.4±11.9% and in control culture to 224±14.7% (* $P < 0.05$ compared to the third day of controls). At 9 days in the presence of dexamethasone, the mast cell numbers decreased to 136±22.5%, still being higher than on the third day (* $P < 0.05$), while in murine mast cells/GR cells the relative number of mast cells increased to 265±20 (** $P < 0.01$). Results are presented as the relative mast cells number (mean±SE) of four experiments done in quadruplicate.

Molecular Analyst Software (Bio-Rad), and the results expressed by relative optical density (OD).

2.4. Immunofluorescence microscopy

Cells were plated on chamber slides and co-cultured for 6 days with or without dexamethasone (10 μg/ml) at 37°C under 5% CO_2. They were fixed with 4% paraformaldehyde (v/v) in phosphate buffer solution for 15 min at room temperature. Primary rat anti-mouse SCF antibody (Genzyme, Cambridge, MA USA), was used at 1:200 dilution for 1 h at 37°C. The secondary rabbit anti-rat antibody (Sigma) was diluted 1:450 and incubated as above. The tertiary goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) was tagged with rhodamine and incubated at 1:400 dilution for 1 h at 37°C. The Golgi complex dye 6-[(*N*-[7-Nitrobenz-2-oxa-1,3-diazol-4-yl]amino)hexanoyl]ceramide (Molecular Probes, Eugene, OR, USA) was used at 10 μg/ml for 5 min. The nuclear dye 4,6-Diamidino-2-phenylindone dihydrochloride (DAPI, Polysciences, Warrington, PA, USA) was used at 0.2 μg/ml in 0.9% NaCl for 5 min at room temperature, as previously described (Mermelstein et al., 1996). Specimens were mounted in glycerol containing 5% (w/v) *n*-propyl gallate, 0.25% 1,4-diazabicyclo 2,2,2 octane, and 0.0025% para-phenylenediamine (all from Sigma). Cells were examined with an Axiovert 100 epifluorescence microscope (Carl Zeiss, Jena, Germany), using filter sets selective for rhodamine, fluoresceine, or the blue wavelength channel. Images were acquired with a C2400i integrated CCD camera (Hamamatsu Photonics, KK, Japan) using Argus processor (Hamamatsu Photonics). Digitized images were transferred to a Dell Optiplex GL 575 computer (Dell Computer, USA). Photographs of processed images were made directly from the monitor screen. Control experiments with no primary antibody or no secondary antibody showed only a faint background staining (data not shown).

2.5. Statistical analysis

Analysis was done using the two tails Mann–Whitney *U* test, and the differences were considered significant at $P < 0.05$.

3. Results

The survival and proliferation of mast cells plated over NIH/3T3 embryo fibroblasts or GR hepatic stellate cells were monitored at the sixth day of co-culture in the presence of increasing quantities of dexamethasone. As previously described (Sakai et al., 1999), the treatment with dexamethasone affected the capacity of NIH/3T3 cells to sustain mast cells, but it did not affect in the same way the GR cells (Fig. 1A–B). The survival and/or proliferation of mast cells were monitored along 9 days of co-culture, in the presence of the highest dexamethasone dose assayed (10 μg/ml). A very strong inhibition of mast cell survival in co-culture NIH/3T3 cells was observed under these conditions. Conversely, no difference was observed in the mast cell survival and proliferation over

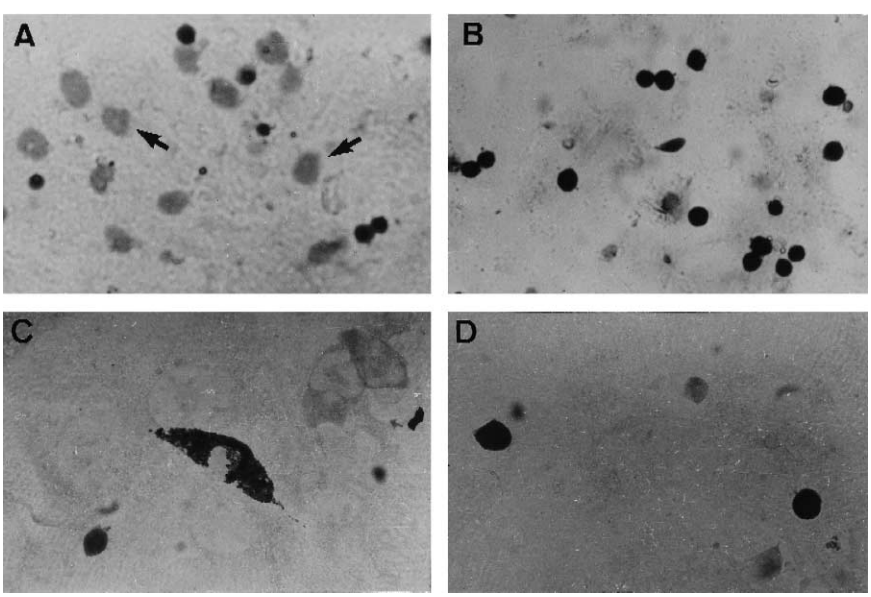

Fig. 3. Murine mast cells in co-culture with GR cells for 6 days without (A) and with (B) dexamethasone (10 μg/ml). In control cultures murine mast cells were spread (arrows), while in co-cultures treated with dexamethasone no spreading was observed. The same results were obtained with co-cultures of mast cells with NIH/3T3 (C and D). Toluidine blue staining; magnification 500×.

GR cells up to the six days of culture. On the ninth day, a small decrease of mast cells was observed, but their total number was still superior to that of the third day (Fig. 2A–B).

The morphological analysis of co-cultures has shown that the spreading of mast cells over the assayed stromas, which occurs normally from the sixth day of culture on, was inhibited by dexamethasone in both experimental models (Fig. 3). The tight cell–cell contact was thus not required for stimulation of mast cells in co-cultures with GR cells.

The potential inhibitory effect of dexamethasone on SCF gene expression was monitored by RT-PCR in NIH/3T3 and GR stroma cells, in the presence of dexamethasone. In view of our previous results indicating that mast cells can induce SCF expression in stellate cells (Brito and Borojevic, 1997), we also monitored the effect of dexamethasone on SCF expression in stromas co-cultured with mast cells. Using the described primer sequences (Flanagan et al., 1991), we obtained the predicted products of amplification under all the culture conditions. These results indicated that both stroma cells showed the message for SCF even when their ability to sustain mast cells was dramatically decreased.

The semi-quantitative RT-PCR was used to assess the regulation of the level of SCF expression under the same culture conditions. The GR cells showed a slightly but not significantly reduced relative expression of SCF in the presence of dexamethasone, but the co-culture with mast cells elicited a large increase of SCF expression, independently of the presence of dexamethasone (Fig. 4A). This result is in agreement with the relatively high number of mast cells observed in the same type of cultures. In NIH/3T3 cells, dexamethasone induced no significant modification of SCF expression, and the presence of mast cells did not induce the up-regulation of SCF, such as observed in GR cells (Fig. 4B). The relative decrease of mast cells during the culture period (from less 10% to 5% of the total cell number) in these cultures could not interfere quantitatively with the observed results.

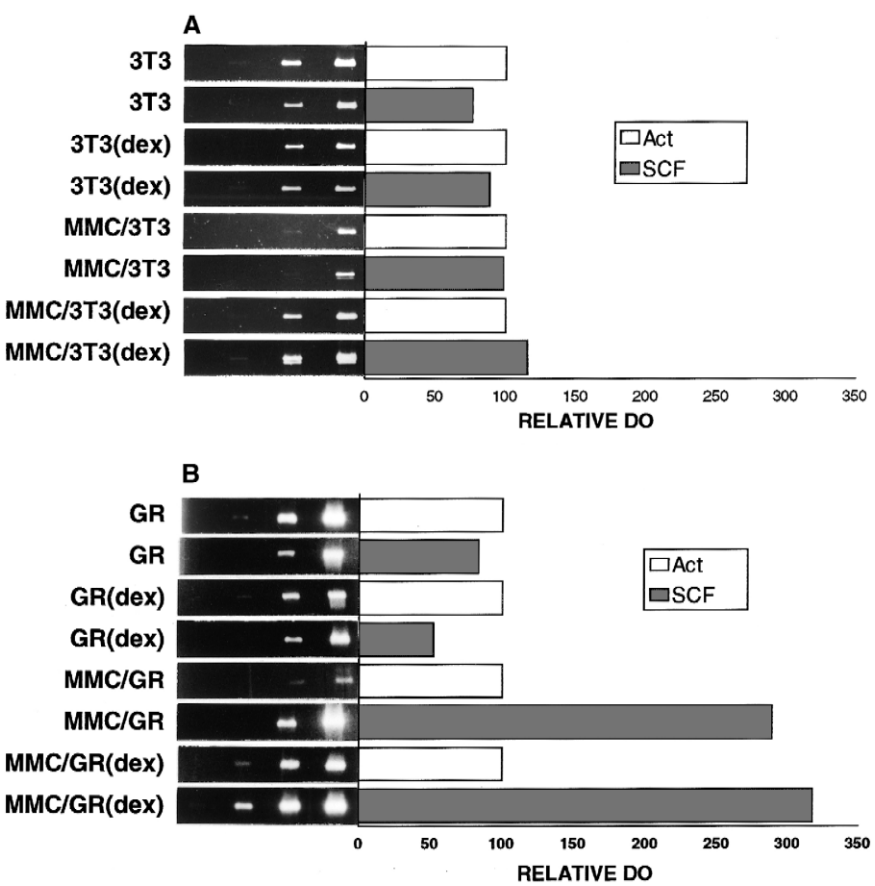

Fig. 4. Semi-quantitative RT-PCR for SCF using cDNA from cultures maintained for 6 days with or without dexamethasone (10 μg/ml). Products of 15, 20, 25 and 30 consecutive amplification cycles are shown. B-actin was used as internal standard. Bands from 25th cycle were analyzed by densitometry, and the results expressed as relative OD. (A) NIH/3T3 cells treated with dexamethasone showed a slight but non-significant increased of the relative OD for SCF when compared to control cultures (88.53% and 76.05%, respectively), as well as NIH/3T3 cells in co-culture with MSC (115.6% and 98.3%, respectively). (B) GR cells showed a reduced relative expression of SCF in presence of dexamethasone as compared to controls (51.6% and 83.1%, respectively). GR cells from in co-culture with murine mast cells showed an up-regulation on relative SCF expression (288.5%), and treatment with dexamethasone did not affect it (317%). Results show a representative RT-PCR experiment, and relative OD for SCF/β-actin were calculated from two independent experiments.

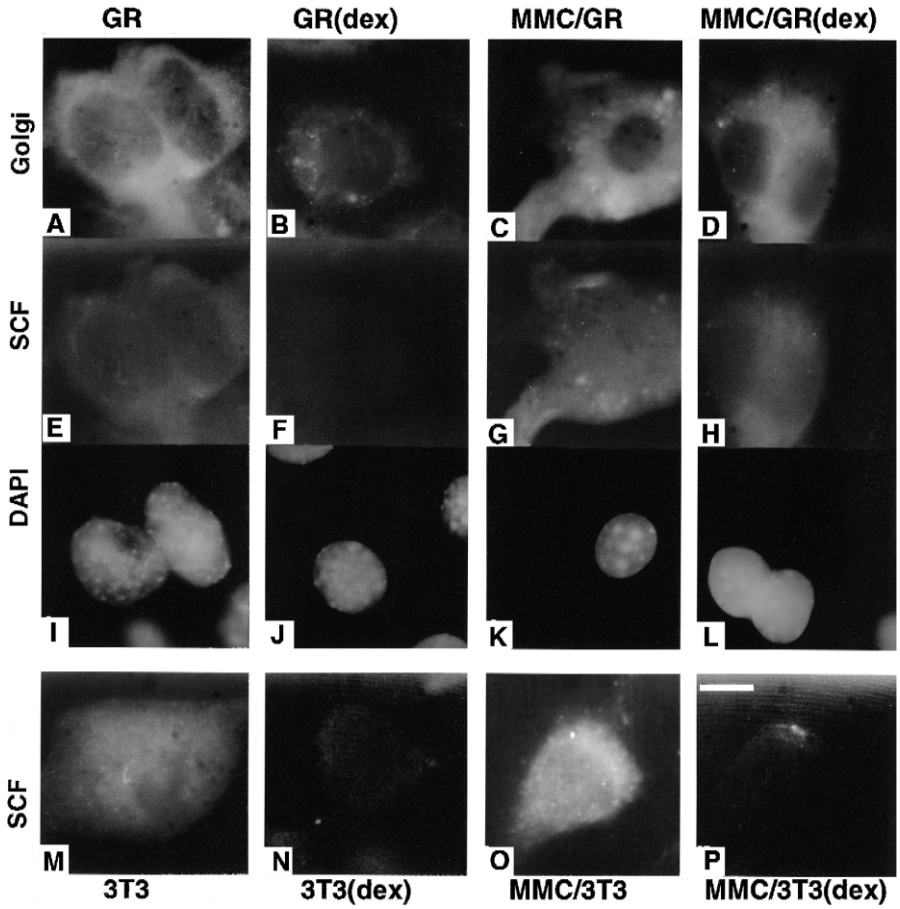

Fig. 5. Immunofluorescence analysis of cellular SCF distribution, NBD-ceramide staining for the Golgi complex and DAPI staining for nuclei in GR and NIH/3T3 cells. Dexamethasone treatment (10 μg/ml) decreased the SCF immunofluorescence in GR cells, NIH/3T3 cells and NIH/3T3 cells co-cultured with MSC (F, N and P) as compared to controls (E, M and O). GR cells from co-culture with murine mast cells showed a strong SCF staining, in controls and in the presence of dexamethasone (H and G, respectively). The Golgi staining was present in all GR cells (A–D) mostly in punctuate staining pattern. GR cells with murine mast cells, with and without dexamethasone showed punctuate staining of Golgi co-localized with SCF (C/G and D/H). Co-localization of SCF staining and Golgi probe was also observed in GR cells alone (A/E). Similar results were obtained in three independent experiments. (Bar = 10 μm).

The fact that NIH/3T3 cells expressed SCF in the presence of dexamethasone, but could not sustain mast cells in co-culture raised the question of the additional post-transcriptional controls of SCF activity. We used immunofluorescence analysis of cellular SCF distribution with antibodies for SCF, together with NCB-ceramide to localize the Golgi complex and the DAPI stain for nuclei. The antibodies against SCF showed a typical membrane-associated protein staining, (Fig. 5E, G, H, M and O), with an occasional intracellular staining localized in the Golgi region (Fig. 5C and D). Dexamethasone treatment decreased the SCF immunofluorescence in both GR and NIH/3T3 cells, as compared with controls (Fig. 5F and N). The very low staining of NIH/3T3 cells co-cultured with mast cells and treated with dexamethasone (Fig. 5P), was in agreement with the low number of mast cells in these cultures. Conversely, mast cells induced in GR cells treated with dexamethasone a strong SCF staining, similar to that in control GR cell (Fig. 5H). Altogether, these results demonstrated that dexamethasone treatment had a post-transcription inhibitory effect on SCF production in both cell types. Mast cells were able to revert fully the dexamethasone effect on GR cells but not on 3T3 cells, indicating a cell type-specific process.

4. Discussion

Our results have shown that dexamethasone has diverse effects on murine skin fibroblasts and hepatic stellate cells, in terms of their interaction with mast cells, and that mast cells can in their turn modulate the dexamethasone effect on the stroma cells.

Dexamethasone inhibited the mast cell spreading on both fibroblasts and stellate cells. It has been shown that mast cells induce the expression of intercellular adhesion molecule (ICAM)-1 and vascular adhesion molecule

(VCAM)-1 in endothelial cells through the tumor necrosis factor-α (TNF)-α release (Meng et al., 1995), and that hepatic stellate cells respond to TNF-α by an increase of the same adhesion molecules (Knittel et al., 1999). On the other side, mast cells increase their β-2 integrin and I-CAM-1 expression through an autocrine loop of IL-4, which may be sensitive to dexamethasone (Babina et al., 1997; Yoshikawa et al., 1999). The rather late spreading of mast cells in co-cultures suggests indeed the progressive induction of the required adhesive mechanisms. Our results indicate that dexamethasone can abrogate these induction pathways, potentially by a direct decrease of the production of inflammatory mediators and in particular of TNF-α in mast cells, which should promote their adhesion and spreading (Choudhury et al., 1996). Alternatively, dexamethasone can interfere with signaling pathways used by pro-inflammatory mediators through dimerization of glucocorticoid receptors with other transcription factors (Reichardt and Schutz, 1998). In contrast to the recent reports on the requirement of mast cell adhesion for their survival, the adhesion was not required for mast cell survival and proliferation in the model of hepatic stellate cell stromas.

Dexamethasone decreased the SCF production in cultures of both stellate cells and fibroblasts. This did not involve the gene expression, but the total protein production as shown by the decreased immunoreactivity in dexamethasone-treated cells. Moreover, the dexamethasone-treated NIH/3T3 cells retained the SCF in the cytoplasm, where it co-localized in part with the Golgi complex (data not shown), inhibiting thus the effect of SCF on the mast cells plated over fibroblasts. The same effect could not be observed in culture of mast cells over hepatic stellate cells, since mast cells reverted completely the dexamethasone effect. This involved simultaneously an increased gene transcription, an enhanced protein synthesis and its translocation to the cell membrane. The mast cell mediators that induce this effect are at present not known. TNF-α is a potential candidate in view of our previous studies (Brito and Borojevic, 1997). In this case the signaling pathways that determine the cell adhesion, which are dexamethasone-sensitive, are different from those that control the SCF synthesis and location.

In conclusion, the tissue background has a relevant role in controls of inflammatory reactions in which mast cells play a pivotal role. Mast cells depend upon the connective tissue stroma for their growth, differentiation and survival, but they can simultaneously modulate the stroma sensitivity to hormones and growth factors. Hepatic stellate cells, which have a number of structural and functional particularities when compared to fibroblasts, have a specific pattern of interaction with mast cells, which may be relevant for understanding of tissue inflammatory reactions in liver. In view of the present results, the liver pathologies potentially related to inflammatory reactions associated with mast cell hyperplasia are not expected to be sensitive to glucocorticoid treatments.

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