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IDENTIFICATION OF GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTOR RECEPTOR mRNA BY NON-ISOTOPIC IN SITU HYBRIDIZATION IN BONE MARROW BIOPSIES

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ABSTRACT

Background. **In the last few years many studies have been published on GM-CSF receptors, focusing on molecular structure, function and distribution. Nevertheless, protocols for detecting GM-CSF receptors on formalin-fixed paraffin-embedded histological sections, to our knowledge, have not been described.**

Methods. **A method based on non-isotopic *in situ* hybridization (ISH) using a 21-base antisense DNA oligoprobe whose 3'-end was labeled with digoxigenin 11-dUTP was devised. The probe was applied on 20 routinely processed bone marrow trephine biopsies which were considered as positive controls.**

Results. **The hybridization signal was seen in myeloid cells, erythroid progenitors and rare megakaryocytes.**

Conclusions. **Non-isotopic ISH represents an alternative to current methodologies for the assessment of GM-CSF receptor expression; since it is suitable for routinely processed samples, it can be regarded as a helpful tool for diagnostic determination of GM-CSF receptors in tumors from patients receiving GM-CSF and for retrospective studies on archival material.**

Key words: GM-CSF receptor, in situ hybridization

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The receptor (R) for human granulocyte-macrophage colony stimulating factor (GM-CSF) is a dimeric molecule involved in the regulation of hematopoiesis, immune and inflammatory responses.[1,2]

In addition to considerable interest in the molecular structure,[3-6] activity,[7-9] and gene localization of GM-CSFR,[10-12] several studies have been published concerning its expression in human normal[13-16] and neoplastic cells.[17-22] Since data from almost all these latter studies were drawn from *in vitro* models, the problem of identifying GM-CSFR in histological section was investigated in the present study. A method based on non-isotopic ISH was devised to detect α subunit (CSFR2A) mRNA in formalin-fixed paraffin-embedded bone marrow trephine biopsies, which were considered a model of routinely processed tissue.

Materials and Methods

Bone marrow samples

Twenty bone marrow trephine biopsies, all performed during 1992, were retrieved from the files of the Department of Biomedical Science and Human Oncology, University of Turin. Histologically, all cases were diagnosed as normocellular bone marrow. Specimens were selected without prior knowledge of the time of fixation and decalcification. Nevertheless, all biopsies were fixed in 10% buffered formalin and decalcified in Osteodec (Bioptica, Milan, Italy) immediately after fixation. All samples were routinely processed and 4 μm sections were cut from each block.

In situ hybridization histochemistry

A 21-base 5' antisense DNA oligoprobe was

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synthesized using an Applied Biosystem Model 391 DNA synthesizer. The unshared portion from residues 171 to 192 (TGTGGTAACT-CACAGAGCAGA) corresponding to Leu8/Pro 14 was selected from cDNA of the α subunit.[4,9] The probe was labeled with digoxigenin 11-dUTP using a DNA 3'-end labeling kit by Boehringer Mannheim Italia (Milan, Italy). Dewaxed sections were washed in TBS, pre-treated with 10 μg/mL proteinase K (Boehringer Mannheim) for 10 minutes at 37°C, post-fixed in 4% paraformaldehyde for 5 minutes at 4°C and washed twice in ice-cold TBS. Sections were incubated with pre-hybridization buffer (50% deionized formamide and 2× SSC) for 15' at room temperature. Hybridization was carried out overnight at room temperature with 30 pmol digoxygenated oligoprobe resuspended in 30 μL hybridization buffer (50% deionized formamide, 6× SSC, 5% dextran sulphate, 50 mM DTT and 0.5% SDS). Washings were done in 4× SSC, 2× SSC at 42°C, 0.5× SSC at 37° C, 0.1× SSC and in TBS at room temperature for 10 minutes each. The hybridization signal was shown by immunohistochemistry employing anti-digoxygenin polyclonal antiserum conjugated with alkaline phosphatase (AP) (Boehringer Mannheim Italia, Milan, Italy) at a dilution of 1:500 for two hours at room temperature. Polyclonal antibody was pre-adsorbed overnight at 4°C with 5% normal human serum in order to reduce unspecific bindings to immunoglobulins. The reaction was developed using NBT/X-phosphate (Boehringer Mannheim) as substrate, and 0.0024 g/mL tetramisole salt (Sigma, St. Louis, USA) was added in order to inhibit endogenous AP.

Specificity controls

Specificity controls[23,24] were carried out as follows.

Human promyelocytic leukemia cell line (HL60) and human lymphocytic leukemia cell line (ST4). ISH was performed on human promyelocytic leukemia (HL60) cells, which are known to express GM-CSF receptors. Cells were cultured according to the protocol reported by DiPersio et al.[3] In addition, a human lymphocytic leukemia (ST4) cell line, which was considered a negative control, was cultured in 1640 RPMI 10% FCS. Cells were fixed in 10% buffered formalin and paraffin embedded according to the celloidin bag procedure.[25]

Northern blot analysis. One × 10^8 HL60 and 1 × 10^8 ST4 cells were used for RNA extraction. Total RNA from HL60 cells was used as positive control, while ST4 total RNA and polyA-RNA served as negative control.

RNA extraction was performed according to the one-step guanidine-thiocyanate procedure.[26] Samples were treated with RNase-free DNase (Boehringer Mannheim) following manufacturer's instructions. RNA concentration was evaluated by spectrophotometry and degradation was monitored by agarose gel electrophoresis. Thirty μg of total RNA from each sample were run on a 50% formaldehyde denaturing gel and transferred to nitrocellulose. Pre-hybridization was carried out at room temperature for 1 hour in 5× SSC containing 50% formamide, 0.02% Sarkosyl, 0.1% SDS and 5% blocking reagent (Boehringer Mannheim). The membrane was hybridized overnight with 30 pmol digoxigenated probe diluted in 5 mL of blocking reagent at room temperature. Washings were performed in 4× SSC, 2× SSC and 1× SSC for 10 minutes at 42°C, and 0.1× SSC for 5 minutes at room temperature. The following steps were carried out with the aid of the Nucleic Acid Detection Kit by Boehringer Mannheim.

Incubation with polyclonal anti-digoxigenin antiserum. One section for each specimen was incubated following the same protocol with omission of the probe.

RNase digestion. One section from each sample was treated for two hours at 37°C with 200 μg/mL DNase-free RNase (Boehringer Mannheim) in 10 mM TRIS-HCL, ph 8, 0.3 M sodium chloride and 0.03 M sodium citrate, and then hybridized as described above.

Competition test. One section from each specimen was hybridized in the presence of 1000-fold unlabeled oligoprobe; another section was hybridized in the presence of 1000-fold 21-base 5' DNA unlabeled oligoprobe for epidermal growth factor (EGF) with an equal content of G and C.

Hybridization with sense oligoprobe. One sec-

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tion for each sample was hybridized with digoxigenated sense CSFR2A oligoprobe following the same protocol.

Results

CSFR2A mRNA was detected in 15 out of the 20 bone marrow biopsies. Thirty per cent of myeloid cells, some erythroid progenitors and a few megakaryocytes showed the cytoplasmic hybridization signal (Figure 1), which consisted of a blue precipitate. Variable signal intensity was seen from biopsy to biopsy in the positive cases. Cytoplasmic staining was uneven in different areas of the same biopsy. In the remaining 5 specimens almost all the cells showed non specific nuclear staining, while the cytoplasm remained unstained.

Formalin-fixed paraffin-embedded HL60 cells displayed the cytoplasmic hybridization signal, while ST4 cells were negative. No staining was seen in sections from bone marrow trephines or HL60 cells incubated with anti-AP polyclonal antibody only, nor in those pretreated with DNase-free RNase, nor in those hybridized in the presence of 1000-fold unlabeled oligoprobe. Hybridization with sense oligoprobe was negative in the 15 positive biopsies, while a non-specific nuclear signal was seen in the remaining 5 specimens. The 1000-fold unlabeled EGF oligoprobe did not compete with the GM-CSFR probe. Northern blot analysis showed a band of approximately 1.2 kb in the lane with HL60, which is consistent with the sequence deposited by Raines et al.[27] No signal was present in the other lanes (Figure 2).

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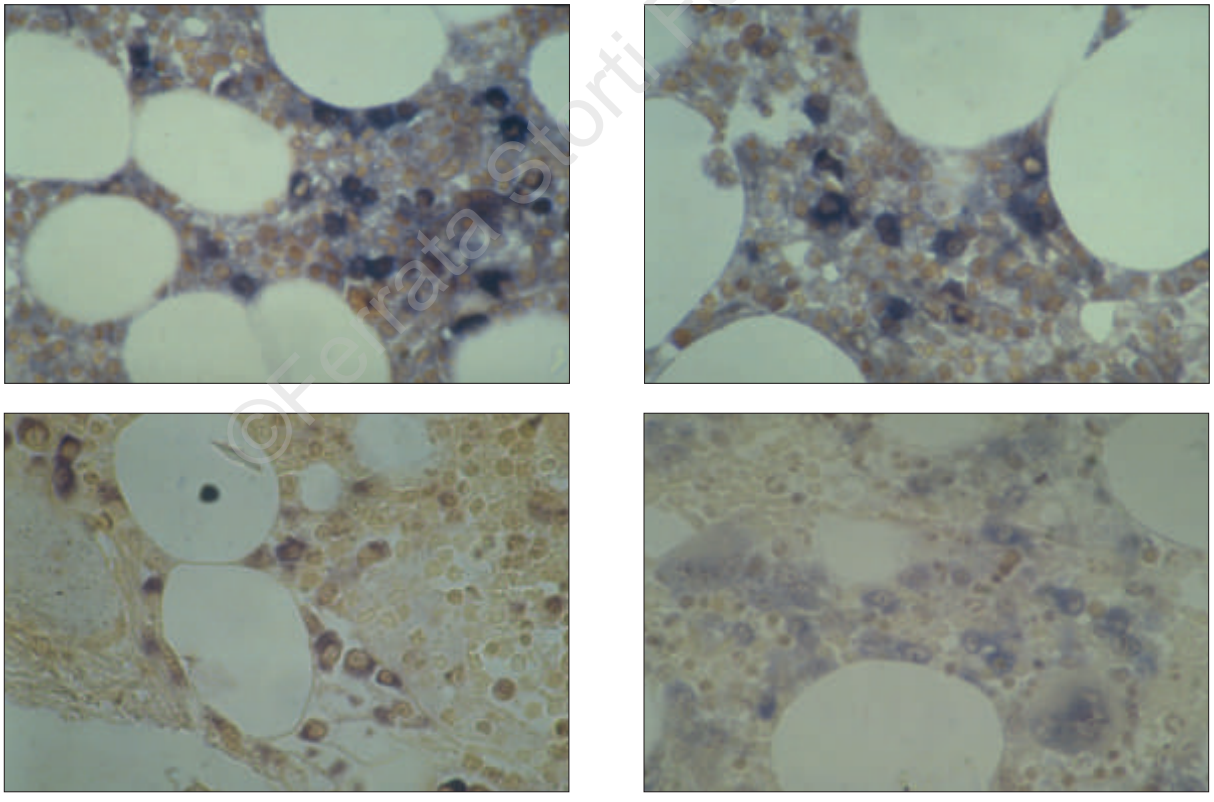

Figure 1. Hybridization signal is present in myeloid progenitors, erythroblasts and megakaryocytes as a blue cytoplasmic precipitate. A variable degree of intensity is shown in four different specimens.

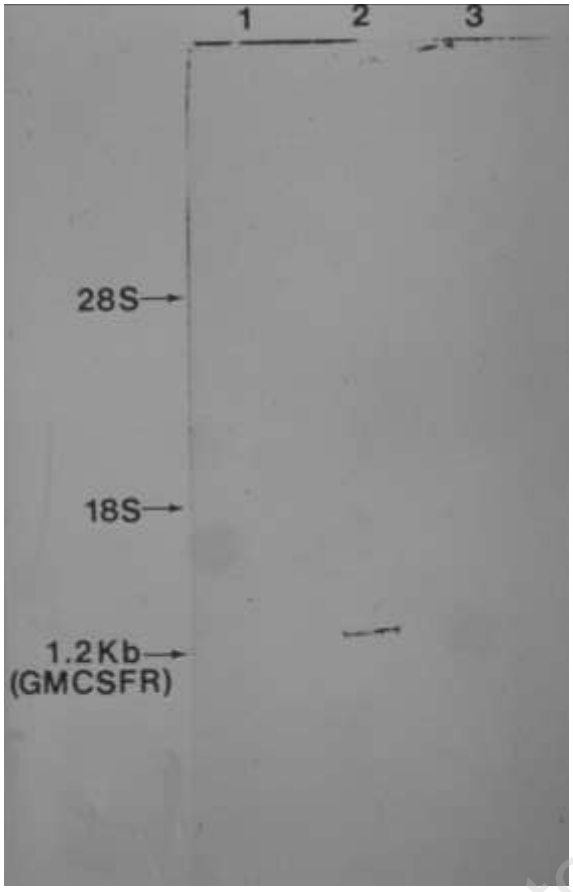

Figure 2. Northern blot analysis was performed in triplicate on human promyelocytic leukemia (HL60) cell line as positive control (lane 2) and human lymphocytic leukemia (ST4) cell line and ribosomal RNA (polyA-) as negative controls (lanes 1 and 3, respectively). Non-isotopic hybridization was carried out with digoxygenated oligoprobe. Hybridization signal is observed in lane 2. Its size corresponds to approximately 1.2 kb.

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Discussion

The protocol based on non-isotopic ISH with antisense oligoprobe presented here appears to be helpful for detecting CSFR2A in histological sections because it allows good morphological identification of receptor-rich cells in formalin-fixed and paraffin-embedded material. Because the α chain plays an essential role in signal transduction and since it has been shown to trigger the signal by itself,[28] its presence was considered a reliable index of GM-CSFR expression.

Bone marrow trephines were considered a good model of histological sections because normal hematopoietic cells constitutively express GM-CSFR. Despite partial loss of RNA in routinely processed decalcified bone marrow biopsies, the hybridization signal was easily detectable in 15 out of the 20 specimens. Since hematopoietic cells were fixed in different steps of their maturation and in different stages of their cycle throughout the same biopsy, the signal was seen in those elements having high levels of CSFR2A mRNA at the time of fixation. In addition, the varying intensity from specimen to specimen may be explained by different degrees of preservation of RNA content. In the remaining 5 biopsies, the absence of a cytoplasmic signal and non specific nuclear staining obtained with sense and antisense probes were interpreted, respectively, as a consequence of RNA extraction and DNA chemical denaturation, both likely due to improper time of fixation and decalcification. Moreover, since bone marrow biopsies are an example of roughly treated material, good results should be expected with any other kind of tissue.

In theory this methodology has two general intrinsic limitations. First, it provides information on the expression of a given mRNA but not on whether mRNA is actually translated, and secondly, the use of an oligoprobe offers a lower amplification compared to full-length probes. Notwithstanding these limitations, non-isotopic ISH should be considered a useful tool of investigation because of the specificity of the oligoprobe employed to distinguish the unshared sequence for CSFR2A from among the similar gene products of cytokine dimeric receptors. The data obtained with controls further confirmed the high specificity of the results.

In light of these considerations, ISH might be an alternative to procedures which allow identification of surface binding sites by linking with labeled GM-CSF or by using monoclonal antibodies in immunocytochemistry (ICC). Those based on rhGM-CSF labeled with ^{125}I,[29] biotin, digoxygenin[30] or phycoerythrin do not work on formalin-fixed tissues. In addition, they may lead to overestimation of results because of the described cross-reactions of GM-CSF with IL-3 and IL-5 receptors,[31] as well as with other

cytokine dimeric binding sites.[32] With regard to ICC, antibodies to the GM-CSF receptor have recently been employed on histological sections of various normal and neoplastic tissues.[33] However, ICC was used on frozen material in which the structure of target proteins should be well preserved. To our knowledge, protocols that work for formalin-fixed tissues, where proteins may be dramatically altered by fixation, have not been published yet.

Finally, the protocol described here might also be a valid adjunct to Northern blot analysis and PCR amplification. The latter show a high degree of sensitivity but since they require tissue homogenization, results can be affected by the presence of different subsets of receptor-rich cells within the same sample.

In conclusion, non-isotopic ISH may help to gain new insights into the expression and distribution of GM-CSFR in normal and neoplastic tissues. Its application to routinely processed biopsies might allow retrospective and prospective studies on neoplastic diseases. It would also have important implications in view of the use of rhGM-CSF in the reconstruction of bone marrow after high-dose chemotherapy-induced aplasia.

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