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Colocalization of tumor necrosis factor-alpha and nitric oxide-synthase immunoreactivity in mast cell granules of nasal mucosa

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Summary. We have demonstrated, with immunohistochemical techniques, the colocalization of tumour necrosis factor-α (TNFα) with a constitutive neuronal isoform of nitric oxide-synthase (NOS) in granules of the majority (52.77%) of the mast cells (MCs) of healthy human nasal mucosa. Very few cells were positive for NOS alone (2.54%). Some cells were positive for TNFα alone (16.73%) or negative for both antigens (18%). Since dim degranulation occurs in MCs of healthy nasal mucosa at any time, we propose that low concentrations of TNFα and NOS secreted by these cells are involved not only in the regulation of homeostasis of normal human nasal mucosa, but also in the survival and function of MCs themselves.

Key words: Mast cells, Nasal mucosa, Nitric oxide-synthase, TNF-alpha

Introduction

Tumor necrosis factor-alpha (TNFα) is a multifunctional cytokine involved in the regulation of tissue homeostasis and local immune responses. Besides macrophages and several leukocytes (Carswell et al., 1975; Cuturi et al., 1987; Dubravech et al., 1990; Higuchi et al., 1990; Costa et al., 1993), mast cells (MCs) have also been recognized as a source of TNFα (Gordon and Galli, 1990). TNFα has been found in MCs of several animal species by pharmacological methods (Young et al., 1987; Richards et al., 1988; Gordon and Galli, 1990). By immunohistochemistry, this cytokine has been localized to the granules of peritoneal MCs of rats and mice (Beil et al., 1994) and in the MCs of the dermis, oral and nasal mucosa of healthy humans (Walsh et al., 1991; Bradding et al., 1995; Walsh, 1995). Besides being stored in granules, TNFα can be synthesized and immediately secreted upon immune stimulation (Young et al., 1987; Old, 1988; Richards et al., 1988; Gordon and Galli, 1990; Benyon et al., 1991).

Nitric oxide (NO) is considered a regulator molecule involved in many cellular functions and in numerous pathologies (see the extensive review by Wolf, 1997). We have recently demonstrated with immunohistochemical techniques that MCs of normal human nasal mucosa contain within their granules a constitutive, neuronal form of nitric oxide-synthase (NOS) (Bacci et al., 1994). NO seems to be a mediator of some effects of TNFα: in particular it increases the cytotoxic effect of this cytokine (Bissonnette et al., 1991). To provide a further insight into the possible functional relationship between TNFα and NO producing enzymes in the nasal mucosa, we have addressed the question as to whether NOS immunoreactivity coexists with that of TNFα in MCs of healthy human nasal mucosa.

Material and methods

Biopsies of nasal mucosa were taken from the edge of the inferior turbinate during surgery for rhinoplasty of 4 men (mean age 24 years, range 18-30 years). The specimens were fixed in Carnoy's solution, dehydrated in ethanol, cleared in xylene and embedded in paraplast. All subjects gave an informed consent and the protocol was carried out following the Italian law and the ethical guidelines of the Italian National Medical Council. For light microscopy, sections were stained with 0.5% toluidine blue (Gurr, Pool, UK) in distilled water. For fluorescence microscopy, sections were treated with tetramethylrhodamine isothiocyanate (TRITC)-labeled avidin (Immunotech, Marseille, France), diluted 1:400, to stain MC granules (Tharp et al., 1985). To study the possible colocalization of TNFα and NOs the following method was used. Paired serial sections were placed apposed on slides, with the lower surface of the first section and the upper one of the second section upside. Both sections of each pair were first stained with TRITC-labeled avidin to stain MC granules. One section

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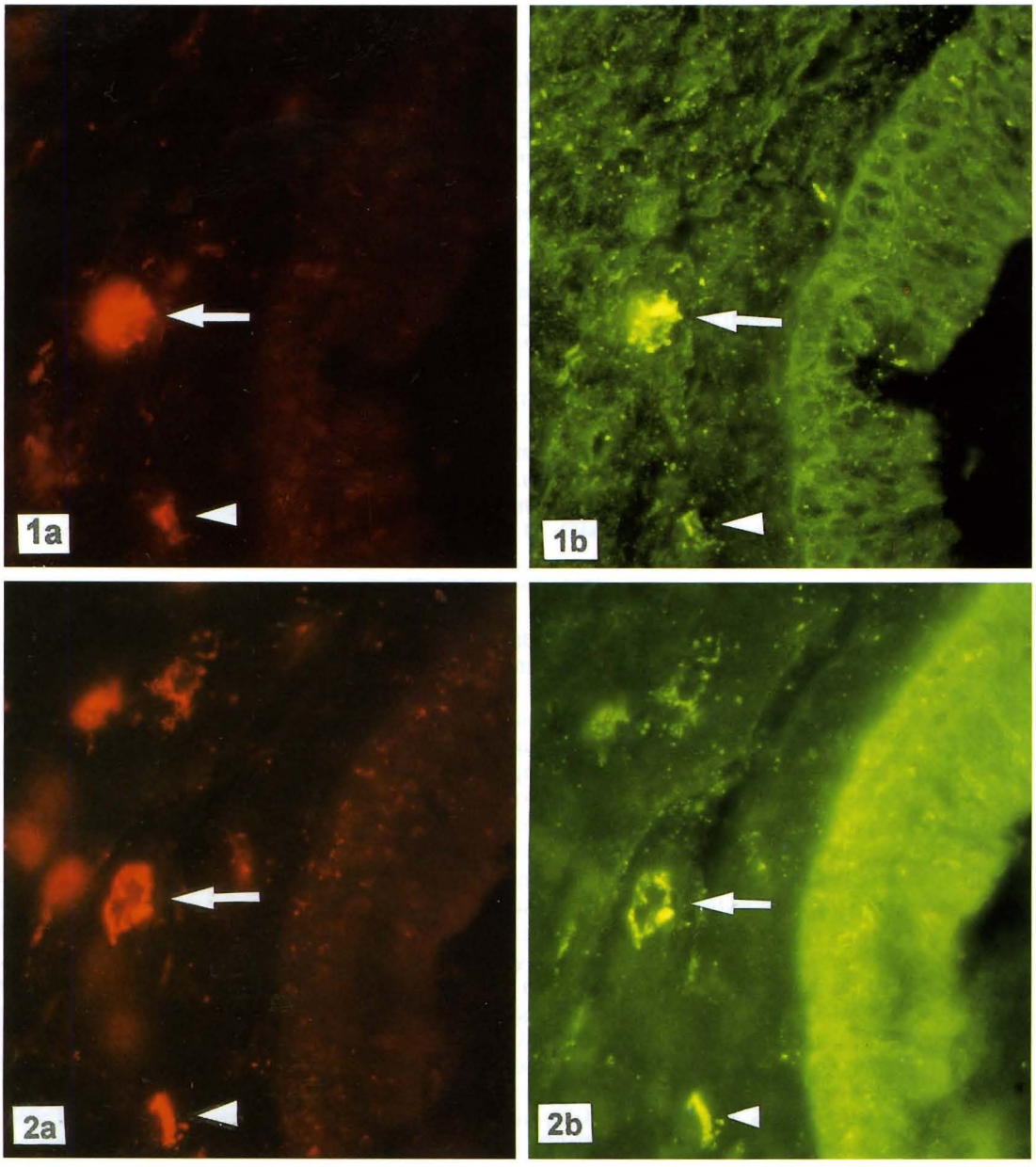
was further immunolabeled with a polyclonal antibody against human TNFα (Sigma, Milan, Italy), diluted 1:50, which was revealed with a fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit polyclonal antibody (Sigma, Milan, Italy), diluted 1:32. In the adjacent section NOS was revealed using a polyclonal antibody, diluted 1:100, followed by fluoresceinated goat anti-rabbit antibody (Sigma, Milan, Italy), diluted 1:32. The antibody for NOS had been raised in rabbits against the enzyme purified from porcine cerebellum and was a kind gift from Dr. B. Mayer (Mayer and Bohme, 1990). The cells labeled with avidin were identified as MCs; those that were recognized in both sections of each pair were used to assess colocalization.

The sections were examined with a Zeiss Axioskop (Heidelberg, FRG) light microscope equipped for epifluorescence. The specificity of the immune staining was tested by omitting the first antibodies or substituting them with irrelevant ones.

For quantitative analysis, each of the four subjects was assumed as a sample unit. Two to six pairs of sections per subject, at least 100 μm apart from each other and observed at magnification x400, were used. The total surface area observed was 2 mm^2 (range 0.3-0.9 mm^2 per subject) and the total number of mast cells observed was 72 (range 8-33 per subject). The number



Figs. 1 and 2. Photomicrographs of two consecutive sections of human nasal mucosa. One section is double-stained with TRITC-avidin, to identify mast cells **(1a)**, and with FITC-conjugated anti-TNF-α antibodies **(1b)** to localize this antigen; the other section is double stained with TRITC-avidin **(2a)** and FITC-conjugated anti-NOS antibodies **(2b)**. Two cells (arrows and arrowheads respectively) are stained in all photomicrographs; the shape is different between figure 1 (a and b) and figure 2 (a and b) because the cells were cut at different levels, but the identity of the cells can be recognized by their position in the context. Fluorescence microscopy. x 400

of cells stained for both TNFα and NOS and those stained for only one antigen were recorded separately for each subject. The results are expressed as mean and standard deviation of the percentage of labeled cells from the total of mast cells.

Results

By light and fluorescence microscopy, MCs appeared to be numerous in the normal human nasal mucosa and their cytoplasm was filled with many granules which stained metachromatically with toluidine blue and were labeled by TRITC-avidin (Figs. 1a, 2a). These cells were scattered in the lamina propria, especially along blood vessels, in the periglandular stroma and in the proximity of the surface epithelium. In paired serial sections the majority of MCs stained for both NOS (Fig. 1b) and TNFα (Fig. 2b) (mean 52.77%, standard deviation 6.75%, range 47.06-62.5%). Fewer MCs were stained for NOS alone (mean 2.54%, standard deviation 3.38%, range 0-7.14%), for TNFα alone (mean 16.73%, standard deviation 19.00%, range 0-42.86%) or for neither antigen (mean 18%, standard deviation 21.46%, range 0-47.06%).

Discussion

To our knowledge, this study shows for the first time that MCs of healthy human nasal mucosa display a colocalization of TNFα and a particular constitutive isoform of neuronal NOs. Since our biopsies were taken from subjects free from any nasal pathology and not treated pharmacologically (except for anesthesia), our results confirm that TNFα is synthesized and stored in granules by the MCs of normal human nasal mucosa even in the absence of stimulation (as shown by Bradding et al., 1995) and that the granular isoform of NOS labeled here is constitutive (Bacci et al., 1994). More important is that these observations also indicate that normal human nasal mucosal MCs are heterogeneous for TNFα and NOS content, since only about approximately a half of the total MCs expressed the immunoreactivity for both molecules.

Since dim degranulation occurs in MCs of healthy nasal mucosa at any time (Rucci et al., 1988), it is reasonable to hyphotesize that low concentrations of TNFα and NOs released by these cells are involved in the homeostasis of the nasal mucosa even in normal conditions. In particular, TNFα might regulate the turnover of adhesion molecules of endothelial cells (Walsh et al., 1991) and the recruitment, terminal differentiation and function of bone-marrow-derived cells, such as dendritic cells and macrophages (Caux et al., 1992; Rossi et al., 1992). On the other hand, the release of NOS could be responsible for the formation of NO in the extracellular matrix and hence induce vasodilation. For this to occur, the necessary co-factor (NADPH) should be contained in the granules, a fact that has yet to be demonstrated. A similar hypothesis has been proposed to explain the functional meaning of the presence of an isoform of NOs within the secretory pathway of endothelial cells (O'Brien et al., 1995). Alternatively, as proposed by Bacci et al. (1994), MCs might secrete this isoform of NOS but not NADPH, in order to sequestrate L-arginine around themselves and thus fix a limit to the possible endogenous production of NO in response to autocrine loops following granule secretion. This, because TNFα induces the production of NO by MCs (Bissonnette et al., 1991) and NO cannot only inhibit MC degranulation (Mannaioni et al., 1991) but can also stimulate the production of other nitrogen radicals presumably involved in cell damage and apoptosis (Buttke and Sandstrom, 1994).

In conclusion, the coexistence of TNFα and NOS within MC granules can lead to a functionally relevant interplay between these two molecules, which could behave as regulating factors for local homeostasis and for the survival and function of MCs themselves.

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