Brief Definitive Report

NEUTROPHIL-ACTIVATING PROPERTIES OF THE MELANOMA GROWTH-STIMULATORY ACTIVITY

By BERNHARD MOSER,* IAN CLARK-LEWIS,§ ROLAND ZWAHLEN,‡ and MARCO BAGGIOLINI*

*From the *Theodor-Kocher Institute, and the ‡Department of Veterinary Pathology, University of Bern, CH-3000 Bern, Switzerland; and the §Biomedical Research Centre, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5*

Melanoma growth-stimulatory activity (MGSA), referring to a peptide of 73 amino acids, was reported to be mitogenic for cultured human melanoma cells (1, 2). Mature MGSA has marked sequence similarity to several recently discovered inflammatory peptides, of which, the human neutrophil-activating peptide 1 (NAP-1/IL-8) was studied most extensively thus far (3, 4). In addition to monocytes, NAP-1/IL-8 was found to be produced by a wide variety of tissue cells, including fibroblasts and endothelial cells, upon stimulation with IL-1 and TNF (3). It induces neutrophil shape changes, chemotaxis, exocytosis of specific and azurophilic granules and the respiratory burst (3) in vitro, and causes exudation and massive neutrophil infiltration in vivo (5). In view of the structural similarities to NAP-1/IL-8 (Fig. 1), it was of particular interest to test MGSA for neutrophil-activating and chemotactic properties. This report shows that MGSA, prepared by chemical synthesis, is a potent inflammatory agonist acting on neutrophils both in vitro as well as in vivo.

Materials and Methods

Materials. Fura-2/AM and BSA were purchased from Fluka AG, Buchs, Switzerland. Human recombinant NAP-1/IL-8 was obtained from the Sandoz Research Institute, Vienna, Austria (6). The sources of other special reagents were described previously (7).

Synthesis of MGSA. MGSA was synthesized by stepwise solid-phase methods on a peptide synthesizer (430A; Applied Biosystems, Inc., Foster City, CA) using double-couple protocols for tBoc amino acids, as described previously (8). After deprotection, purification, and folding (9), homogeneity of the material was analyzed by reverse-phase HPLC and IEF (Fig. 2). Amino acid analysis gave the composition expected, and the M_r of 7,857 daltons, deduced by mass spectroscopy, was consistent with the calculated value of 7,859 daltons for MGSA.

Cell Preparation. Human neutrophils were isolated from buffy coats of donor blood (7). The final suspension consisting of 10^8 cells/ml in 0.15 M NaCl supplemented with 0.05 mM $CaCl_2$ was kept at 10°C until use.

Biological Assays. Elastase release (7, 10), H_2O_2 production (11), neutrophil chemotaxis (12), and cytosolic-free calcium changes (13) were determined according to established methods.

In Vivo Activity. Adult male Wistar rats were given intradermal injections of NAP-1/IL-8 or MGSA (10^{-9}, 10^{-10}, 10^{-11}, or 10^{-12} mol/site) in 0.05 ml pyrogen-free saline. Full-thickness skin samples were fixed in 4% formalin, embedded in paraffin, stained with hematoxylin and eosin, and examined by light microscopy (5).

Address correspondence to Bernhard Moser, Theodor-Kocher Institute, University of Bern, P. O. Box 99, CH-3000 Bern 9, Switzerland.

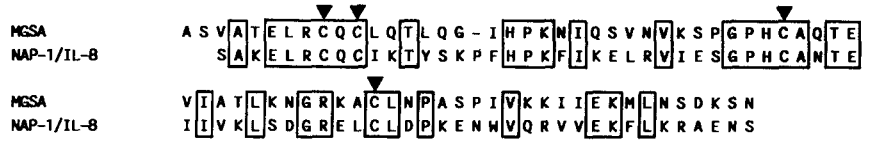

FIGURE 1. Structural similarities between MGSA and NAP-1/IL-8. The proteins were aligned according to their four cysteine residues (*arrow heads*). Conserved amino acids are boxed.

Results

Activation of Human Neutrophils. The effect of MGSA in inducing exocytosis as assessed by the release of elastase from azurophil granules was concentration dependent and similar to that of NAP-1/IL-8 (Fig. 3 *a*). Both peptides were effective in the same molar range and elicited a response at the threshold concentration of 3×10^{-10} M. NAP-1/IL-8 was somewhat more potent (25–35%) than MGSA throughout the range of concentrations tested. NAP-1/IL-8 also induced a rapid and transient respiratory burst response at concentrations of 10^{-9} to 10^{-7} M, confirming former results (6). By contrast, MGSA showed only borderline activity (Fig. 3 *b*). At 10^{-7} M, the highest concentration tested, MGSA-stimulated neutrophils produced <2% (<10 pmol) of the H_2O_2 obtained with NAP-1/IL-8.

The data summarized in Table I demonstrate that MGSA is chemotactic as well. Both MGSA and NAP-1/IL-8 induced neutrophil migration in the concentration range of 10^{-10} to 10^{-7} M, with maximal effects at 10^{-8} M. As seen in exocytosis, MGSA was slightly less potent as a chemoattractant than NAP-1/IL-8.

Like classical chemotactic agonists, such as C5a and fMet-Leu-Phe, MGSA induced a transient rise in the concentration of cytosolic-free calcium ($[Ca^{2+}]_i$). Calcium mobilization was observed at concentrations as low as 10^{-10} M, and a maximal transient elevation was reached at 10^{-8} M (Fig. 4, *inset*). The curves in Fig. 4, representing the maximal rates in raising $[Ca^{2+}]_i$ as a function of MGSA or NAP-1/IL-8 concentration, were virtually identical. The rates in mobilizing cytosolic-free calcium did not reach a plateau in the range of concentrations tested.

In Vivo Effects of MGSA. Light microscopy of skin samples taken 4 h after in-

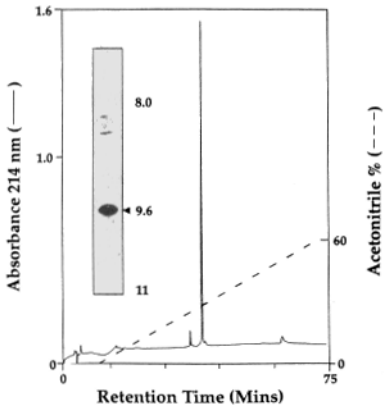

FIGURE 2. Analysis of purified synthetic MGSA. 15 μl of the purified peptide (1 mg/ml) was loaded onto a Vydac C-18 reverse-phase HPLC column and eluted with a 0–60% water-acetonitrile gradient. The inset represents a silver-stained IEF gel of purified MGSA. The gel was equilibrated in 8 M urea, 2% Ampholines pH 8–11, then run and stained using the "PHAST" electrophoresis system (Pharmacia Fine Chemicals, Piscataway, NJ). The pH gradient was determined using a surface pH electrode and positions corresponding to pH 8.0 and 11.0 are indicated. The pI of MGSA was estimated to be 9.6 (*arrow head*).

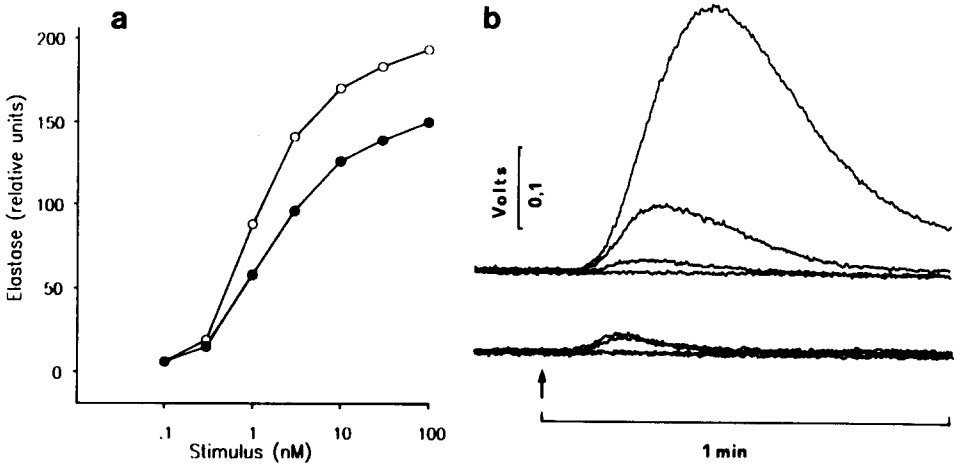

FIGURE 3. (*a*) Release of elastase from cytochalasin B-treated human neutrophils stimulated with MGSA (●) or NAP-1/IL-8 (○). Elastase activity is expressed in relative fluorescence units (1 U = 1 pMol 7-amino-4-methylcoumarine produced/min/10^6 cell) (10). (*b*) Production of H_2O_2 by neutrophils after stimulation with MGSA or NAP-1/IL-8. The rate of H_2O_2 production is proportional to the chemiluminescence intensity (volts) (11). In the experiment shown, the maximal rate of H_2O_2 production with 10^{-7} M NAP-1/IL-8 was ~50 pMol/s/10^6 cells. NAP-1/IL-8 (*top*) and MGSA (*bottom*) were tested at 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M (top to bottom). Similar values were obtained in two (*a*) and one (*b*) additional experiments performed with different cell preparations.

tradermal application of MGSA at 10^{-9} mol/site demonstrated a massive infiltration of neutrophils (Fig. 5). No other granulocytes, monocytes, or lymphocytes were detected. The infiltration was most prominent around venules in the deeper dermal layers (Fig. 5 *a*), but extended downwards into the subcutis and upwards into the upper dermis. Significant responses of decreasing intensities were obtained with 10^{-10}, 10^{-11}, and 10^{-12} mol/site (data not shown).

Discussion

This study shows that synthetic MGSA activates and is chemotactic for human neutrophils in vitro and induces massive neutrophil infiltration in vivo. Except for the respiratory burst response, its potency is similar to that of NAP-1/IL-8.

TABLE I

Neutrophil Chemotaxis Induced by MGSA and NAP-1/IL-8

Stimuli	NAP-1/IL-8	MGSA
nm		
0.1	1.67 ± 0.18	1.30 ± 0.13
1.0	2.02 ± 0.08	1.72 ± 0.10
10	2.07 ± 0.09	1.83 ± 0.12
100	1.85 ± 0.29	1.76 ± 0.13

Numbers refer to chemotactic indices (mean ± SD; n = 3). 2 × 10^5 cells/Boyden chamber were used (12); random migration in the absence of stimuli amounted to 3.63 ± 0.44 × 10^4 cells.

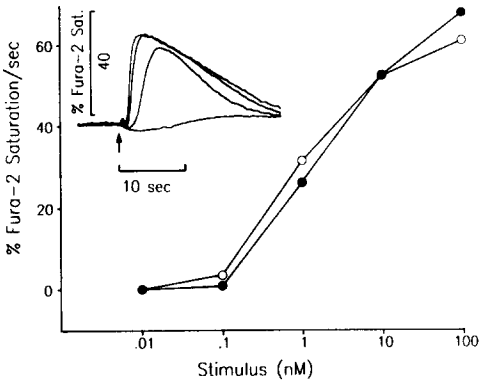

FIGURE 4. Rate of cytosolic calcium rise in human neutrophils stimulated with MGSA (●) and NAP-1/IL-8 (○). Values represent the mean of two separate experiments. The inset shows the transient changes in $[Ca^{2+}]_i$ expressed in percent Fura-2 saturation after stimulation with MGSA. Resting and maximal level of $[Ca^{2+}]_i$ were estimated to be 0.1 and 0.6 μM, respectively (13). The curves, from top to bottom, correspond to 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M MGSA. Addition of the stimulus at time zero is indicated by the arrow.

MGSA and NAP-1/IL-8 were prepared by unrelated methods, stepwise chemical synthesis for the former, and expression in *Escherichia coli* of a synthetic gene for the latter (6). Chemical synthesis has established itself as an alternative approach to recombinant technologies for the production of biologically active polypeptides of considerable size, such as IL-3 (8, 9) and the human granulocyte-macrophage CSF (14). The MGSA preparation was found to be of high purity, and it thus appears unlikely that the effects reported here are due to synthetic byproducts.

The in vitro and in vivo effects reported in this study indicate that MGSA may function as an inflammatory mediator by mechanisms similar to those postulated for NAP-1/IL-8 (3). Recent findings showing that the gene coding for MGSA (*gro*/MGSA) is expressed in several types of tissue cells supports this hypothesis.

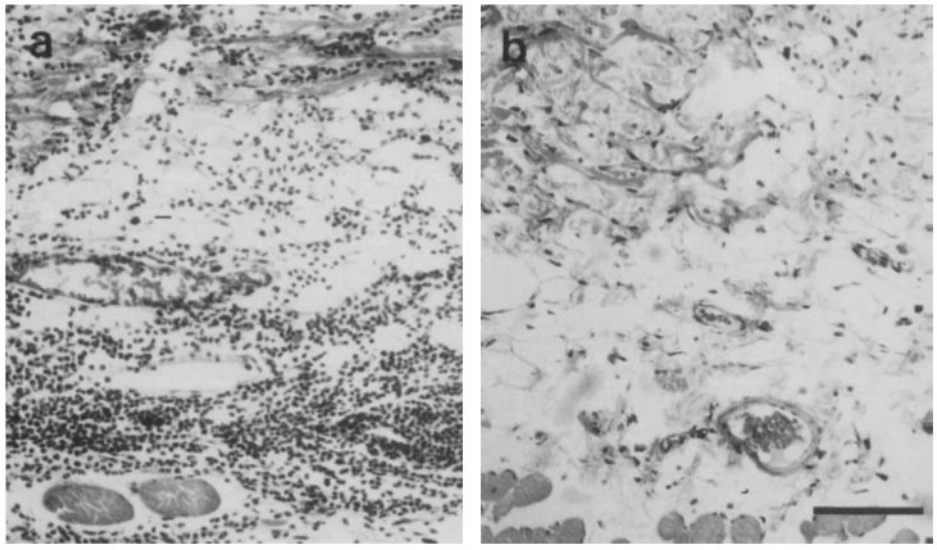

FIGURE 5. Histology of rat lower dermis 4 h after intradermal injections of 10^{-9} mol MGSA. Skin samples were processed as described in Materials and Methods. (*a*) MGSA; (*b*) saline control. Solid bar represents 100 μm.

Anisowicz et al. (15) demonstrated by Northern blot analysis that the *gro*/MGSA mRNA levels are considerably elevated in human fibroblasts and mammary epithelial cells exposed to inflammatory stimuli such as IL-1 or phorbol 12-myristate 13-acetate. In addition, Wen et al. (16) reported the induction of *gro*/MGSA expression in human endothelial cells after stimulation with TNF, IL-1, or LPS.

Richmond et al. (1) identified MGSA on the basis of its mitogenic activity for Hs294T human melanoma cells. Biochemical and immunocytochemical studies on human melanoma biopsies suggested that MGSA is involved in maintenance of tumor progression. The present study demonstrating the potent neutrophil-activating properties of MGSA indicates that this peptide, in addition to its reported mitogenic activity, may function as a mediator of inflammation.

Summary

Melanoma growth-stimulatory activity (MGSA), a peptide reported to be mitogenic for Hs294T human melanoma cells, has extensive sequence similarity to the neutrophil-activating peptide NAP-1/IL-8, suggesting functional similarities. To test this hypothesis, MGSA was chemically synthesized and tested for its effects on human neutrophils. It was found to induce chemotaxis, exocytosis of elastase, and changes in cytosolic-free calcium to an extent and at concentrations similar to NAP-1/IL-8. However, MGSA was considerably less potent than NAP-1/IL-8 in inducing the respiratory burst. Intradermal injections in rats of MGSA resulted in a massive accumulation of neutrophils. Our data demonstrate that, apart from its growth-stimulatory activity, MGSA is a potent inflammatory agonist with neutrophil-stimulating properties.

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