Volume 282, number 2, 373–376 FEBS 09704 May 1991

ADONIS 001457939100411C

Effects of neutrophil adherence on the characteristics of receptors for tumor necrosis factor-α

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Received 11 March 1991

Human recombinant [^{125}I]TNF-α was incubated with non-adherent human neutrophils, cells adherent to fibronectin-coated plastic, or adherent cells scraped into suspension (post-adherent). Binding of TNF to all cells increased with doses of added TNF but adherent cells bound little TNF. Binding of TNF by post-adherent cells was greater than when adherent, but still significantly less than that of non-adhered neutrophils, suggesting that TNF receptors were relocated on the adherent surface of neutrophils. Scatchard analysis showed that adherent cells expressed significantly fewer TNF receptors, but of higher affinity, than non-adherent cells. The results suggest that altered expression of TNF receptors might contribute to the differential effects of TNF on adherent and non-adherent neutrophils.

Neutrophil; Tumor necrosis factor; Receptor

1. INTRODUCTION

Tumor necrosis factor-α (TNF) is one of several cytokines that can affect the functions of human neutrophils. Neutrophils in suspension may be induced, by TNF, to adhere to a variety of substrates [1–3], but some effects of TNF are only apparent on neutrophils that are already adherent. Previous evidence has suggested that the production of superoxides, release of lactoferrin, and proteolysis of extracellular protein by neutrophils in vitro is increased significantly by TNF only if the cells are allowed to adhere first [2–7]. The reasons for this heterogeneity in neutrophil response to TNF are unclear. It could be due to changes in the nature of the TNF receptors following cell adherence, or altered post-receptor events. Previous studies of TNF binding by neutrophils have suggested that these cells express a single population of TNF receptors [8–11] but it is not clear whether the state of the cell can influence the expression of TNF receptors and hence the response of the neutrophil to this cytokine. The purpose of this study was to investigate whether adherence of neutrophils results in altered expression of TNF receptors.

2. MATERIALS AND METHODS

2.1. *Isolation of blood neutrophils*

Venous blood from five healthy volunteers was collected into lithium heparin anticoagulant. Neutrophils were isolated by centrifugation on Percoll density gradients [12]. Each blood sample was diluted with an equal volume of 0.15 mol/l NaCl solution and layered carefully onto 2 ml of 1.075 g/ml Percoll (Pharmacia AB, Uppsala, Sweden) in 0.15 mol/l NaCl, which had been layered over 3 ml of 1.096 g/ml Percoll. The tubes were centrifuged at 400 × g for 25 min and the neutrophils harvested from the interface between the two Percoll layers. The cells were washed twice in Tris-buffered RPMI 1640 medium (Flow Laboratories, Rickmansworth, UK), counted and resuspended in medium. The cells were >96% neutrophils and viability, assessed by exclusion of Trypan blue, was >98%. All reagents were assayed for endotoxin using the Kabi Vitrum Coatest (Flow Labs) and contained less than 20 ng/l.

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2.2. *Binding of TNF*

Human [^{125}I]TNF (400–800 Ci/mmol) was obtained from Amersham International (UK) and pure, recombinant human TNF was kindly supplied by Dr. G.R. Adolf (Ernst-Boehringer Institut für Arzneimittel-Forschung, Austria). The neutrophils from each subject were treated in three ways for TNF binding studies.

(i) Non-adhered neutrophils. Neutrophils (10^6) were incubated, with agitation, for 30 min in 0.1 ml of 'binding medium'; RPMI 1640 with 0.1 g/ml bovine serum albumin, 5 mg/ml cytochalasin B and 0.65 mg/ml sodium azide (all reagents from Sigma Chemical Co., Dorset, UK). These cells were dispensed to microfuge tubes and incubated (in triplicate) with [^{125}I]TNF at concentrations ranging from 0.015–0.5 nmol/l. Control preparations also contained unlabelled TNF at 1000-fold the concentrations of [^{125}I]TNF. The cell suspensions were rotated continuously for 1 h at 4°C and then 0.1 ml phthalate oil (1.5 vol. di-*n*-butyl-phthalate/1 vol. di-iso-octyl-phthalate; from BDH Chemicals, Dorset, UK) was added to each tube, which was spun for 2 min in a microfuge and frozen to −70°C. Each tube was cut at the oil layer and the cells pellets and supernatants assayed separately for [^{125}I]TNF using an LKB Multigammacounter. The number of receptors/cell and dissociation constant (K_d) were calculated by Scatchard analysis [13].

(ii) Adherent neutrophils. Linbro tissue culture plates were loaded with 1 ml of 10 mg/ml human fibronection (Sigma Chem. Co.) dissolved in 0.15 mol/l NaCl. After 1 h, the solution was removed and the plates allowed to dry. Neutrophils (10^7 in 1 ml RPMI 1640) were added to the fibronectin-coated wells and incubated for 1 h at 37°C

in an atmosphere of 5% CO_2/95% air. Non-adherent cells were removed by washing with medium and counted in order to calculate numbers of adherent cells. To the adherent cells, [^{125}I]TNF was added as described above. After incubation, the medium in each well was collected and spun through phthalate oil. The supernatant (free ligand) and the 'pellet', representing any detached neutrophils, were collected. The adherent cells in the Linbro dishes were lysed by the addition of 2% sodium dodecylsulphate and the lysate added to the 'pellet' to obtain total 'bound' TNF. Binding of TNF to these cell preparations was insufficient for Scatchard analysis.

(iii) Post-adherent neutrophils. Adherent neutrophils were prepared as described above. Non-adherent cells were removed by washing with RPMI 1640. Adherent cells were incubated for 30 min with binding medium (see above), harvested with a rubber policeman, counted and resuspended in the same medium containing [^{125}I]TNF (0.015-0.5 nmol/l). Bound and free [^{125}I]TNF measurements and Scatchard analysis were performed as described above for non-adhered cells.

2.3. *In situ localization*

Neutrophils (2×10^6) in suspension were incubated with 0.5 nmol/l [^{125}I]TNF, as described above, for 1 h at 4°C and unbound ligand removed by repeated washing. The cells were resuspended in binding medium, cytospun and the slides air-dried, coated with NTB emulsion (Kodak), dried at room temperature for 5 min and stored in a light-tight dessicator at 4°C. After 7-14 days, the slides were dipped sequentially in: Kodak D19 developer (2.5 min), 1% acetic acid (1 min), Kodak fixer (5 min), distilled water (3 x 10 min), all at 15°C. After air drying, the slides were counterstained with Diff-Quik (Travenol Labs) and examined microscopically for silver grains denoting binding of [^{125}I]TNF.

3. RESULTS

Fig. 1 shows the average amounts of TNF bound, with increasing concentrations of [^{125}I]TNF, by the three sets of neutrophil preparations from five subjects. All cells bound TNF in a dose-dependent manner. The neutrophils in suspension bound significantly more TNF than adherent cells but increased binding was observed by adherent (post-adherent) cells after removal from substrate. In the presence of 1000-fold excess of unlabelled TNF, binding of [^{125}I]TNF was $<2\%$ of that observed in the absence of unlabelled TNF.

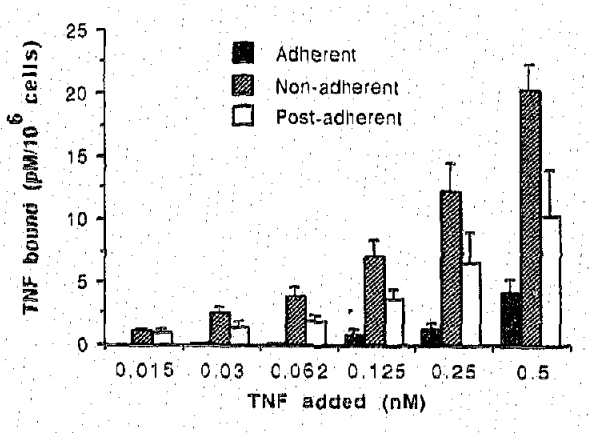

Fig. 1. Binding of [^{125}I]TNF to isolated human neutrophils in suspension (non-adherent), adhered to fibronection-coated plastic (adherent) and after removal from substrate (post-adherent). Results are mean values (±SEM) of measurements obtained with neutrophils from five different donors.

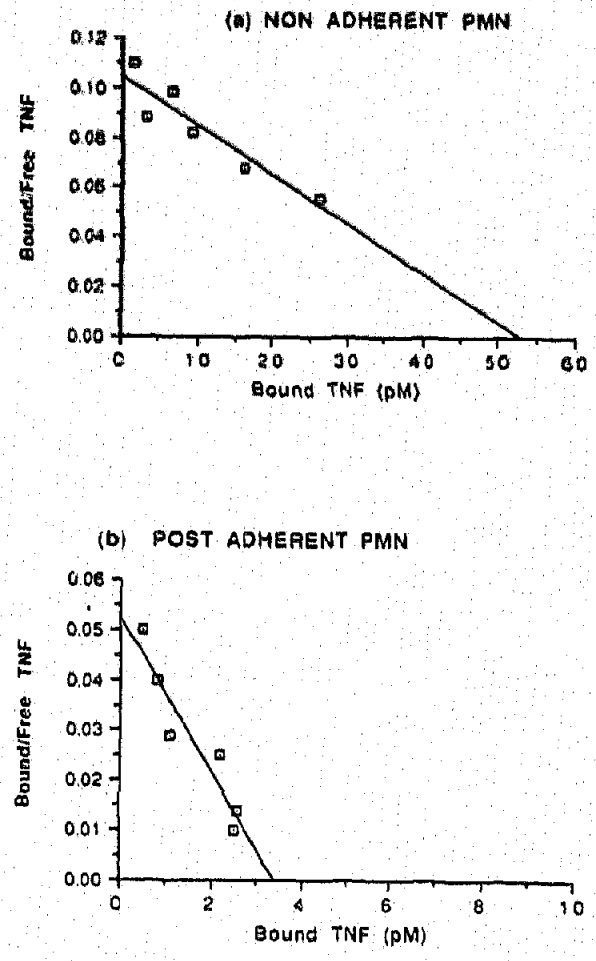

Fig. 2. Examples of Scatchard plots of TNF binding by (a) non-adherent and (b) adherent neutrophils from one donor.

Scatchard analysis of the results from the adherent neutrophils was not possible because of the small amounts of [^{125}I]TNF bound by these cells. Fig. 2 shows examples of Scatchard plots of non-adhered and post-adherent cells from one individual. The average numbers of TNF receptors and K_d, calculated from the Scatchard plots of neutrophils from the five subjects are shown in Fig. 3. The non-adherent neutrophils expressed significantly more ($2P<0.015$) TNF receptors (mean 1212; SEM 488 receptors/cell) than the post-adherent cells (mean 547; SEM 454 receptors/cell). The affinity of the TNF receptors on the non-adherent cells was significantly ($2P<0.001$) lower (mean K_d 0.28; SEM 0.07 nM) than that of the post-adherent neutrophils (mean K_d 0.12; SEM 0.03 nM).

In situ localization demonstrated, visually, the binding of [^{125}I]TNF to isolated neutrophils, but occasional eosinophils were negative (Fig. 4).

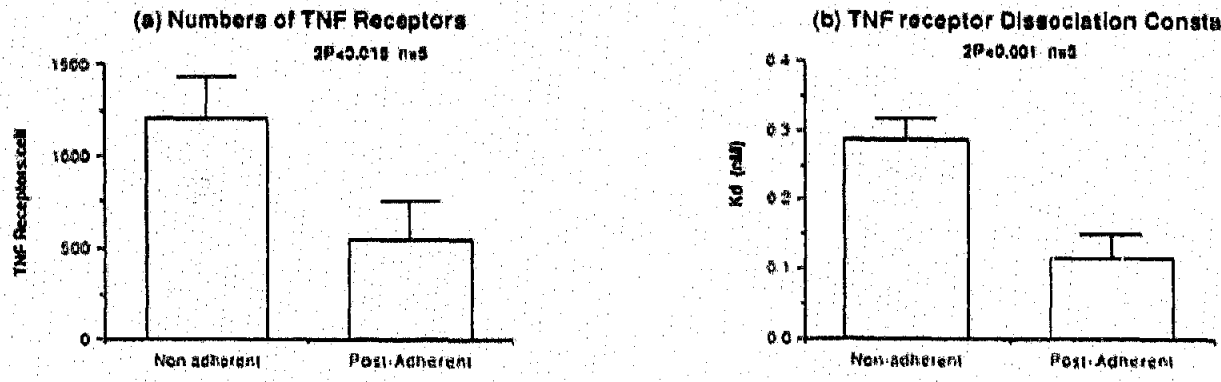

Fig. 3. Average numbers (± SEM) of (a) TNF receptors/cell and (b) dissociation constants calculated from Scatchard analysis of binding studies of neutrophils from five donors.

4. DISCUSSION

Previous studies have partially characterised the nature of receptors for TNF on human neutrophils [8–11]. The reported number of receptors/cell (400–6000) and K_d ($0.3–13 \times 10^{-10}$ M) represent a wide range. The results of the present study confirm the presence of high affinity receptors on human neutrophils, but indicate that the nature of the cells (adherent or in suspension) is crucial in understanding the nature of the TNF receptors. The in situ experiments failed to demonstrate binding of TNF to eosinophils in our cell preparations. This suggests that eosinophils might lack TNF receptors although further

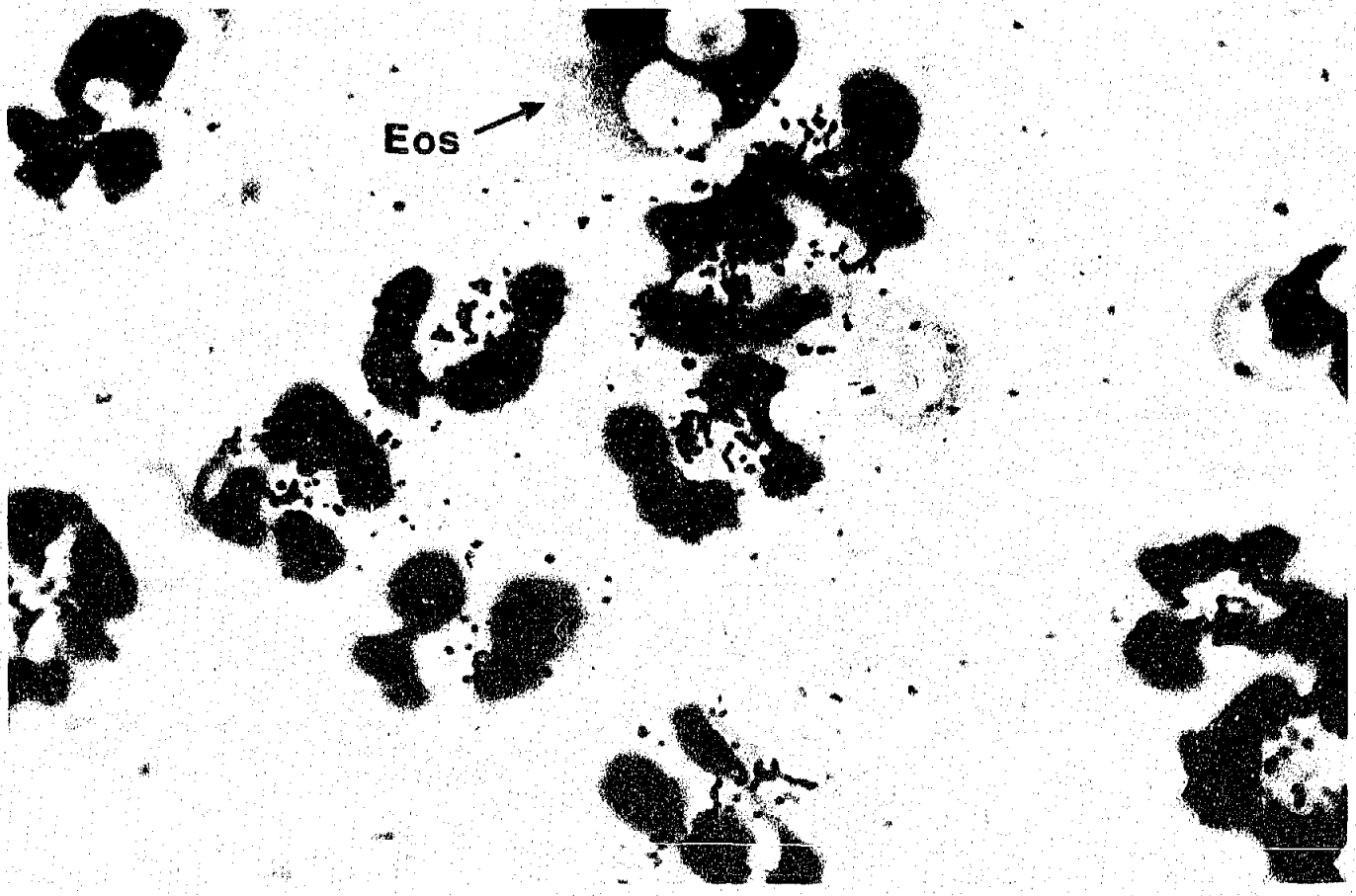

Fig. 4. In situ localization of binding of [^{125}I]TNF to a cytospin preparation of human neutrophils. Silver grains show binding of TNF. An eosinophil (Eos) is negative.

experiments would be necessary to establish whether this is the case.

Our initial measurements resulted in a significant loss of binding of TNF to neutrophils when the cells were adhered. We consequently investigated binding by the adherent cells after physical removal from the substrate, with a subsequent recovery of binding of TNF by what we have termed 'post-adherent' neutrophils. These results suggest that, when neutrophils adhere, receptors for TNF are located largely on the adherent surface, thus restricting access to the ligand in solution around the cell. Scatchard analysis indicated that the numbers of receptors on these cells are fewer than on those which have not adhered and that their affinity is greater. Polarization of neutrophil membrane receptors is not unprecedented; receptors for C3b, Fc and formyl peptides have been reported to redistribute to the anterior surface of migrating neutrophils [14,15]. It is unclear at present whether the change in expression of TNF receptors when neutrophils adhere represents only the loss, by internalization or shedding, of most receptors except those on the adherent surface. The change in affinity of TNF receptors on the adherent cells, however, suggests that they could represent a new population of molecules. Two TNF receptors on myeloid cell lines have been studied in some detail to date; a receptor of 75 kDa (Type A) and another of 55 kDa (Type B) [16,17]. The relationship of these receptors to those expressed by neutrophils will require further investigation.

The results of several studies have suggested that the effects of TNF on neutrophil behaviour are limited, resulting in cell adherence but not in the activation of other neutrophil functions such as the production of superoxides and release of granule contents [1-7]. The latter effects appear to be restricted to adherent neutrophils. In the present study, we have investigated the possibility that the nature of the response of neutrophils to TNF might be modulated by the expression of TNF receptors. The results suggest that this may be the case. Clearly, further studies will be required in order to establish a definite association between alterations in TNF receptors and neutrophil response to TNF.

The physiological significance of such a controlling mechanism is clear. Neutrophils in the blood which encounter TNF in the regions of inflamed tissues where cytokines are released would be induced to adhere to endothelium, but without a stimulation of potentially harmful effects such as the release of superoxides and proteolytic enzymes. The latter effects would be consequent upon the initial adherence of the cells and their migration into the affected tissues. The different responses of adherent and non-adherent neutrophils are, furthermore, not confined to TNF but have been observed with other cytokines [3-5,18]. It is therefore possible that the response of neutrophils to a wide range of mediators is controlled by the altered expression of surface receptors.

Acknowledgements: This work was supported by the Chest, Heart and Stroke Association, the TSB Trust and the British Lung Foundation.

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