GLUCOCORTICOID HORMONE SUPPRESSION OF HUMAN NEUTROPHIL-MEDIATED TUMOR CELL CYTOSTASIS

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In the present study, we have investigated the effect of glucocorticoid hormones on neutrophil-mediated tumor cell cytostasis and found that hydrocortisone and a synthetic hormone, dexamethasone (Dex), inhibited cytostasis in the presence or absence of tumor necrosis factor-α. The effect of Dex was completely reversed by a glucocorticoid receptor antagonist, RU38486. To clarify the underlying mechanisms, we examined effects of Dex on the binding avidity of β2 integrin on the neutrophil surface and how these might in turn affect neutrophil-to-tumor cell binding. Dex was found to inhibit these neutrophil properties, and RU38486 completely suppressed both forms of Dex inhibition. Taken together, our findings suggest that glucocorticoid hormone inhibition of neutrophil-mediated tumor cell cytostasis is at least partially due to a lowering of the ligand binding avidity of β2 integrin on the neutrophil surface. Int. J. Cancer 81:74–80, 1999.

The primary importance of neutrophils resides in their role as effector cells in host defenses against bacterial infections. Furthermore, these cells are cytotoxic toward tumor cells under certain conditions, especially when treated with various immunomodulators (Inoue and Sendo, 1983). Neutrophils also play an important role in the modulation of the immune response (Tamura et al., 1994). In contrast to these beneficial effects, neutrophils may harm normal host tissues in certain pathological conditions such as non-RELOF syndrome (Shiga et al., 1991) and septic hepatic necrosis (Sato et al., 1993). In such situations, control of neutrophil function may be useful in increasing the beneficial activities of these cells, i.e., tumor cell cytotoxicity, and in suppressing their harmful activities. In a series of experiments to study the role of neutrophils in a variety of biological and pathological circumstances using a monoclonal antibody (MAB) that selectively depletes neutrophils in vivo (Sekiya et al., 1989), we found that neutrophil apoptosis is modulated differently depending on the conditions employed, such as in vivo infiltration into inflammatory foci (Tsuchida et al., 1995). While observing neutrophil apoptosis regulation, we unexpectedly found that neutrophils obtained from volunteer donors under stressful conditions exhibited a low sensitivity to apoptosis (Sendo et al., 1997).

To clarify the underlying mechanisms of this phenomenon, we examined the effects of various stress hormones in vitro, and found that hydrocortisone and dexamethasone (Dex) inhibit both spontaneous and tumor necrosis factor (TNF)-α-induced human neutrophil apoptosis (Kato et al., 1995). To further explore the effects of glucocorticoids on neutrophil functions, we examined their effects on neutrophil-mediated tumor cell cytostasis (NMTCC), and found that hydrocortisone and Dex are both capable of NMTCC inhibition.

MATERIAL AND METHODS

Tumor cell lines

We used KG-1 (a human acute myeloblastic leukemia), Daudi (a Burkitt’s lymphoma) and KMT-17 (a methylcholanthrene-induced fibrosarcoma of WKA rats) as target tumor cells in the NMTCC assay. These tumor cell lines were maintained in RPMI-1640 medium (GIBCO, Chagrin Falls, OH) supplemented with 10% heat-inactivated fetal calf serum (FCS, Whittaker, Walkersville, MD), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mg/ml NaHCO₃ and 3 mg/ml HEPES (Dojin, Kumamoto, Japan).

Reagents and media

Recombinant human TNF-α (rhTNF-α) was generously provided by Dainippon (Tokyo, Japan); Dex, progesterone and hydrocortisone were purchased from Wako Junyakukogyo (Osaka, Japan) and RU38486 (Moguilewsky and Philibert, 1984) was a gift from Dr. D. Philibert (Roussel-UCLAF, Romainville, France). The latter 3 reagents were dissolved initially in ethanol and diluted subsequently in media. The final ethanol concentration in the media were less than 0.01%. Adrenocorticotropic hormone (ACTH) was purchased from SeikagakuKogyo (Tokyo, Japan) and dissolved in phosphate-buffered saline (PBS). Other reagents and culture media used in this study were purchased from commercial sources as indicated in brackets: dextran (Midorijuji, Osaka, Japan), Ficoll Paque (specific gravity; 1.077; Pharmacia, Uppsala, Sweden) and RPMI 1640 (GIBCO, Grand Island, NY). Mouse anti-human CD11a (MHM24), CD11b (2LPM19c), CD11c (KB90), CD18 (MHM23) and a negative control MAB (X0931) were purchased from DAKO (Glostrup, Denmark).

Neutrophil purification, isolation and culture in vitro

Neutrophils were separated from the peripheral blood of a normal human volunteer. Heparized peripheral venous blood was added to 2 vol of dextran and this mixture was kept at room temperature for 60–90 min. The supernatant containing leukocytes was collected and washed with PBS. The cell suspensions were then centrifuged at 400 g for 30 min at room temperature on Ficoll Paque, and a pellet containing neutrophils and red blood cells was obtained. After red blood cells had been removed by hypotonic shock through sequential use of 0.2% and 1.6% NaCl solutions, the pellet was used as a neutrophil source. The purity of neutrophils was greater than 96%, as determined by Giemsa staining.

NMTCC assay

Neutrophils were incubated in the presence or absence of rhTNF-α (concentration: 10 U/ml) for 30 min at 37°C. In some experiments, they were further treated with various MABs for 30 min at 4°C. Neutrophils (2 × 10⁵/well) in RPMI-1640 medium supplemented with 10% FCS were then incubated with tumor cells (2 × 10⁵/well) in the presence or absence of various hormone solutions, in wells of a NuncWell 96-well culture plate (Falconn 3072, Oxnard, CA), adjusting the total volume in each well to 200 µl. The control group consisted of tumor cells in the presence of the medium alone. The reaction mixtures were incubated for varying periods of time at 37°C in 5% CO₂ and 95% air. Tumor cell proliferation was assayed by measuring the incorporation of [³H]-TdR (1 µCi/well) (10 Ci mmol, specific activity; ICN, Irvine, CA) during the final 6 hr of culture, as described in detail elsewhere (Inoue and Sendo, 1983). The cells were then harvested with Minimash (Laboscience, Tokyo, Japan). The samples were dried on
filter papers, placed into scintillation liquid (ACS; Amersham, Aylesbury, UK), and counted with a scintillation counter (LSC751; Aloca, Tokyo, Japan). All assays were performed in triplicate. The percentage of uptake inhibition was determined by the following formula:

\[ \text{% uptake inhibition} = (1 - \frac{\text{cpm of experimental group}}{\text{cpm of tumor cells in the presence of the medium alone}}) \times 100. \]

**EiC3b rosette assay**

We followed the method of Iida et al. (1987). Briefly, to obtain C3b, C3 purified from fresh human serum was incubated with factors B and D. Sensitized sheep erythrocytes (EA) were incubated sequentially with Clgp, C4hu and oxidized C2hu to prepare factors B and D. Sensitized sheep erythrocytes (EA) were incubated with iC3b and were used as EiC3b. Purified neutrophils (4 x 10^5) at 37°C for varying periods of time. Percentages of uptake were determined, as described in Materials and Methods.

\[ \text{% percentage inhibition} = \text{(rosette-forming neutrophils/number of neutrophils)} \times 100. \]

**Flow cytometric analysis**

After a 30-min incubation of neutrophils in the presence or absence of 10^{-7} M of various hormones at 37°C with or without a 30-min pretreatment with TNF-α (10 U/ml), these cells were collected, washed with PBS/0.05% NaN3/1% BSA and incubated with 10 µg/ml of various antibodies in PBS/0.05% NaN3/1% BSA. This reaction was performed on ice for 30 min. After washing twice with PBS/0.05% NaN3/1% BSA, the cells were incubated for 30 min on ice with fluorescein isothiocyanate (FITC)-conjugated albumin (BSA). The tubes were rotated for 30 min at 37°C, and rosette-forming cells were counted under microscopy. Cells bearing more than 2 sheep red blood cells (SRBC) were considered to be rosette-forming cells. More than 100 neutrophils were scanned in the presence of the medium alone.

**TABLE I - EFFECTS OF HORMONES ON SPONTANEOUS AND TNF-α-INDUCED NMTCC**

| Hormone (10^{-7} M) | TNF-α (10 U/ml)-treated | TNF-α (10 U/ml)-treated | TNF-α (10 U/ml)-treated |
|---------------------|-------------------------|-------------------------|-------------------------|
|                     | KG-1                    | Daupi                   | KMT-17                  |
| Medium alone        | 67.2 ± 4.3              | 78.3 ± 2.9              | 62.6 ± 3.9              | 70.4 ± 2.9              | 61.7 ± 2.8              | 71.6 ± 3.4              |
| ACTH                | 65.5 ± 3.9              | 76.4 ± 4.3              | 61.4 ± 1.5              | 71.1 ± 3.2              | 62.7 ± 4.1              | 70.1 ± 4.0              |
| Epinephrine         | 66.1 ± 3.5              | 77.4 ± 2.9              | 60.6 ± 2.5              | 70.6 ± 3.0              | 59.1 ± 3.2              | 69.4 ± 1.2              |
| Progesterone        | 65.8 ± 3.5              | 76.0 ± 3.9              | 61.9 ± 2.3              | 69.4 ± 1.9              | 62.0 ± 3.0              | 68.6 ± 2.3              |
| Hydrocortisone      | 36.0 ± 2.9              | 46.1 ± 3.6              | 35.0 ± 3.6              | 44.0 ± 2.8              | 34.2 ± 2.1              | 43.1 ± 4.1              |
| Dexamethasone       | 32.4 ± 3.5              | 44.7 ± 3.4              | 33.1 ± 2.5              | 42.6 ± 3.5              | 32.6 ± 3.6              | 42.5 ± 2.3              |

TNF, tumor necrosis factor; NMTCC, neutrophil-mediated tumor cell cytostasis; ACTH, adrenocorticotropic hormone. Medium or TNF-α (10 U/ml)-treated neutrophils were incubated with target tumor cells, KG-1, Daupi and KMT-17 for 18 hr in the presence or absence of various steroid hormones. Results are expressed as mean ± SE of 3 separate experiments. *p < 0.05 compared with the medium only group.

**FIGURE 1** - Kinetics of dexamethasone (Dex)-induced neutrophil-mediated tumor cell cytostasis inhibition. Untreated or tumor necrosis factor (TNF)-α (10 U/ml)-stimulated neutrophils were incubated with the target tumor cells, KG-1, in the presence or absence of Dex (10^{-7} M) at 37°C for varying periods of time. Percentages of uptake inhibition were determined, as described in Material and Methods. □: medium alone; ■: Dex 10^{-7} M; ○: TNF-α 10 U/ml; ●: TNF-α 10 U/ml + Dex 10^{-7} M. Results are expressed as means ± SE of 3 separate experiments. *p < 0.05 compared with the medium only group; #p < 0.05 compared with the TNF-α-treated group.

**FIGURE 2** - Requirement for early addition of dexamethasone (Dex) for neutrophil-mediated tumor cell cytostasis inhibition by this glucocorticoid. Untreated or tumor necrosis factor (TNF)-α (10 U/ml)-treated neutrophils were incubated with the target tumor cells, KG-1, for 18 hr. Dex (10^{-7} M) was added at 0, 15, 30, 60 and 90 min of incubation, as shown in Material and Methods. □: medium alone; ●: Dex 10^{-7} M; ○: TNF-α 10 U/ml; ■: TNF-α 10 U/ml + Dex 10^{-7} M. Results are expressed as means ± SE of 3 separate experiments. *p < 0.05 between Dex-treated and untreated groups.
antimouse Ig (DAKO F0313), which was diluted to 1:20 with PBS/0.05% NaNO₃/1% BSA. The cells were washed twice, fixed with 1% paraformaldehyde-PBS and analyzed with a FACSCalibur (Beckton Dickinson, Mountain View, CA).

In vitro neutrophil-to-tumor cell adhesion assay

Neutrophils were incubated at 37°C for 1 hr in the presence of 700, 10, 6 and 0.1 μM sodium-51Cr-chromate adjusted with FCS to a total volume of 1.2 ml. 51Cr-labeled neutrophils were stimulated or not stimulated with TNF-α (10 U/ml) for 30 min, and neutrophils (2 × 10⁶/well) were then incubated in 96-well plates coated with KG-1 tumor cells in the presence or absence of Dex and RU38486. After various periods of time, non-adherent labeled neutrophils were washed out, and the remaining cells were lysed with 0.1 N HCl. The supernatants were counted with a gamma counter.

Statistical methods

Statistical analysis was performed with a t-test. A p value below 0.05 was taken as indicating a significant difference.

RESULTS

Effect of various hormones on NMTCC

To determine whether the endocrine system exerts any influence on NMTCC, we examined the effect of various hormones on spontaneous and TNF-α-induced NMTCC using agents such as ACTH, epinephrine, Dex, hydrocortisone and progesterone. As shown in Table I, human neutrophils inhibited [³H]-TdR uptake of 3 different target tumor cells, KG-1, Daudi and KMT-17, both in groups treated or not treated with TNF-α. Significantly higher cytostasis was observed in TNF-α-treated groups compared with untreated groups. The addition of hydrocortisone and Dex significantly inhibited spontaneous and TNF-α-induced NMTCC, but addition of the other hormones did not.

We then examined the effects of concentration. At concentrations of 10⁻⁷ M and 10⁻⁶ M, which are relevant to human serum levels of corticosteroid hormones, Dex inhibited spontaneous and TNF-α-induced NMTCC (data not shown). We next studied the time course of spontaneous and TNF-α-induced NMTCC as well as Dex-induced inhibition, using KG-1 as target tumor cells. Spontaneous and TNF-α-induced NMTCC increased up to 18 hr incubation, and thereafter started to decline. A similar time course was observed in the groups subjected to further treatment with Dex. Both spontaneous and TNF-α-induced NMTCC were inhibited significantly at 12, 18 and 24 hr incubation, and spontaneous NMTCC was further inhibited at 30 hr incubation (Fig. 1). We then examined what effect the timing of Dex addition had on inhibition of NMTCC. We added Dex at 0, 15, 30, 60 and 90 min of incubation, and incubated the sample for a total 18 hr prior to assessing NMTCC inhibition. Figure 2 shows that Dex inhibited spontaneous and TNF-α-induced NMTCC only when it was added to the culture at the beginning of the incubation, suggesting that Dex is effective at inhibiting NMTCC in the very early phase of the reaction.
FIGURE 4 – Effects of various hormones on neutrophil β2 integrin expression. (a) After 30 min incubation at 37°C in the presence or absence of 10^{-7} M of various hormones, neutrophils were treated with each of the antibodies on ice for 30 min. These cells were then incubated for 30 min on ice with fluorescein isothiocyanate (FITC)-conjugated antimouse Ig sera. Neutrophils fixed with 1% paraformaldehyde were analyzed with a FACS Calibur. (b) After 30 min incubation in the presence or absence of tumor necrosis factor (TNF)-α, neutrophils were incubated with each of the antibodies on ice for 30 min, followed by 30 min on ice with FITC-conjugated antimouse Ig sera. Neutrophils fixed with 1% paraformaldehyde were analyzed with a FACS Calibur. Arrow shows CD11b and CD18 expression on TNF-α-treated neutrophils.
teroids modulate the expression of CAM and its ligand binding avidity (Cronstein et al., 1992). We thus hypothesized that inhibition of NMTCC by Dex might have resulted from CAM modulation by this hormone. To test this hypothesis, we first examined whether CAMs are involved in NMTCC. Based on earlier studies (Barnett et al., 1995), we decided to examine the effect of adding MAbs capable of blocking β2 integrins activity. The MAbs to CD18, CD11b and CD11c, but not to CD11a, partially inhibited spontaneous and TNF-α-induced NMTCC (Fig. 3), suggesting that both these NMTCC processes are at least partially mediated through Mac-1 and/or p150/95. Secondly, we examined whether there was any change in β2 integrin expression on neutrophil surfaces as a result of treatment with various hormones. As shown in Figure 4a, CD18 expression was reduced significantly by treatment with Dex and hydrocortisone for 30 min at 37°C. However, that of CD11a, CD11b or CD11c was unchanged. No change in β2 integrin expression was induced by treatment with ACTH or epinephrine. TNF-α slightly but significantly enhanced expression of CD11b and CD18 (Fig. 4b; arrow) but not that of CD11a or CD11c (Fig. 4b). These slightly augmented levels of expression were not reduced by Dex treatment. Because expression of β2 integrin does not necessarily parallel ligand binding avidity (Schwartz et al., 1995), we examined the effects of Dex on the binding avidity of β2 integrin to iC3b, ligand of Mac-1 and p150/95, using the EiC3b rosette assay. As shown in Figure 5, treatment of neutrophils with TNF-α enhanced the percentage of rosette forming cells. The percentage was reduced by Dex treatment in both TNF-α-treated and untreated groups (Fig. 5). RU38486 abrogated this reduction, indicating that Dex exerts its effect through the glucocorticoid receptor.

**DISCUSSION**

We have shown that the glucocorticoid hormones, hydrocortisone and Dex, inhibit spontaneous and TNF-α-induced NMTCC, thus enhancing information on the immunomodulation by glucocorticoid hormones. The effects of corticosteroid hormones on neutrophils have not been studied as systematically as those on lymphocytes, and earlier findings remain controversial (Losito et al., 1978; Perretti and Flower, 1993). Glucocorticoid hormones inhibit neutrophil transendothelial migration in vivo (Perretti and Flower, 1993). Furthermore, in vivo administration of glucocorticoid hormones transiently enhances the number of peripheral blood neutrophils (Dale et al., 1975). On the other hand, adhesion and surface expression of L-selectin and CD18 on neutrophils are profoundly inhibited by in vivo administration of glucocorticoid (Burton et al., 1995). Considering the above findings on transendothelial migration and adherence in conjunction with our findings that Dex reduced iC3b binding of neutrophils (Fig. 5), glucocorticoid inhibition of NMTCC may be at least partially due to the reduced binding of neutrophils to target tumor cells via β2 integrin. In fact, interaction between β2 integrin on neutrophil surface and its ligand, ICAM-1, on endothelial cells has been demonstrated to be essential for neutrophil-mediated cytotoxicity against the endothe-

**FIGURE 5** – Dexamethasone (Dex) suppression of the ligand binding avidity of neutrophil β2 integrin and its restoration by a glucocorticoid receptor antagonist, RU38486. Neutrophils treated (a) or not treated (b) with tumor necrosis factor (TNF)-α (10 U/ml) for 30 min at 37°C, were incubated with EiC3b for varying periods of time in the presence or absence of 10^{-7} M Dex and RU38486. Neutrophils binding more than 2 EiC3b cells were regarded as rosette-positive cells. □ Medium; △: Dex 10^{-7} M; ○: Dex 10^{-7} M + RU38486 10^{-6} M. Results are expressed as means ± SE of 3 separate experiments. *p < 0.05 compared with the Dex-untreated group.
integrin reflects not only the strength of expression of this protein but also its molecular configuration on the cell surface. Some MAbs to integrin may recognize epitopes within the vicinity of the ligand binding region, but others may not. In our present experiment, the MAb to CD18 may have recognized an epitope located in an area close to the neutrophil-to-target tumor cell binding site, but the MAbs directed to CD11b and CD11c may have not. This may be the reason why Dex treatment reduced CD18 expression alone (Fig. 4). Even if this supposition is correct, the fact that Dex failed to reduce the slight augmentation in TNF-α-induced CD18 expression is difficult to explain using the above speculation. Although actual mechanisms remain unclear, the molecular configuration of β2 integrin on the neutrophil surface might have been changed by TNF-α-treatment, resulting in the development of resistance to Dex by hiding from the surface the epitope recognized by the MAb to CD18. However, inasmuch as iC3b binding of neutrophils was reduced by Dex treatment, this epitope may not be directly involved in ligand binding. Further use of MAbs to epitopes of β2

**FIGURE 6** – Dexamethasone (Dex) suppression of neutrophil adhesion to tumor cells and its restoration by a glucocorticoid receptor antagonist receptor, RU38486. Neutrophils were incubated at 37°C for 1 hr after the addition of 51Cr. 51Cr-labeled neutrophils were stimulated (a) or not stimulated (b) with tumor necrosis factor (TNF)-α (10 U/ml) for 30 min, then, 2 × 10⁶/well of these cells were incubated in a 96-well plate coated with KG-1 tumor cells in the presence or absence of Dex and RU38486. After various periods of time, non-adherent labeled neutrophils were removed and the remaining cells were lysed with 0.1 N HCl. The supernatants were counted in a gamma counter. □: Medium; Δ: Dex 10⁻⁷ M; ○: Dex 10⁻⁷ M + RU38486 10⁻⁶ M. Results are expressed as means ± SE of 3 separate experiments. *p < 0.05 compared with the Dex-untreated group.

**FIGURE 7** – Suppression of neutrophil adhesion to tumor cells by monoclonal antibodies (MAbs) to β2 integrin. Neutrophils were incubated at 37°C for 1 hr after the addition of 51Cr. 51Cr-labeled neutrophils were stimulated (a) or not stimulated (b) with tumor necrosis factor (TNF)-α (10 U/ml) for 30 min, and then, treated with each MAb for 30 min at 4°C; 2 × 10⁶/well of these cells were incubated in a 96-well plate coated with KG-1 tumor cells. After various periods of time, non-adherent labeled neutrophils were removed and the remaining cells were lysed with 0.1 N HCl. The supernatants were counted in a gamma counter. □: negative control; ○: CD11a; △: CD11b; ▪: CD11c; ■: CD18. Results are expressed as means ± SE of 3 separate experiments. *p < 0.05 compared with negative control group.
integrin on activated leukocytes (Petruzelli et al., 1995) may clarify these situations.

Our present finding that glucocorticoids suppress NMTCC validates the argument for the immunosuppressive effects of these hormones on immune reactions mediated by neutrophils. As mentioned above, some reports have demonstrated that glucocorticoids do not affect neutrophil functions such as superoxide production, phagocytosis or bacterial killing (Losito et al., 1978). However, other reports, as well as our present findings, clearly show that glucocorticoids inhibit β2 integrin-mediated adhesion and tumor cell cytostasis, and induce transient neutrophilia (Burton et al., 1995; Dale et al., 1975). These types of activities lead us to speculate that glucocorticoids affect some neutrophil functions but not others. During a previous study in which we found that glucocorticoids inhibit neutrophil apoptosis and lengthen the survival of this cell, we speculated that these hormones might affect neutrophil functions in a positive manner inasmuch as an increase in the neutrophil population might enhance host defense by optimizing the number of neutrophils engaged in killing bacteria (Kato et al., 1995). However, our present results on the inhibitory effects of glucocorticoids on tumor cytostasis, as well as reports on the complex effects of these hormones on the modulation of sepsis (Molijn et al., 1995) suggest that further systematic observations are required for determining the true effects of glucocorticoids on neutrophil functions in cancer patients, especially because glucocorticoids are used frequently in treatment of certain cancers, and neutrophils may be possible effector cells in cancer immunotherapy (Tanaka and Sendo, 1993).

As for the mechanisms involved in glucocorticoid hormone-mediated NMTCC, further work is needed on gene transcription dependency of the observed effect, because some important effects of glucocorticoid hormones are indeed independent from DNA binding of the glucocorticoid receptor (Reichardt et al., 1998).

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