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A POSSIBLE ROLE OF GLUCOCORTICOIDS: AN INTRINSIC INHIBITOR OF THE CYTOTOXIC ACTIVITY OF TUMOR NECROSIS FACTOR

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The cytotoxic activity of tumor necrosis factor (TNF) against L929 fibroblasts *in vivo* was noncompetitively inhibited by physiological concentrations of glucocorticoids such as hydrocortisone ($10^{-7}M$), corticosterone ($5\times10^{-8}M$) and dexamethasone ($5\times10^{-9}M$). The inhibition was abolished by the addition of actinomycin D (0.5 μg/ml) or cycloheximide (4μM). A phospholipase A_2 inhibitor, quinacrine ($2\times10^{-6}M$), also inhibited the TNF cytotoxicity. These findings suggest that the antitumor cytotoxic reaction by TNF is regulated by glucocorticoid through some mechanism involving *de novo* transcription and translation and that this regulatory mechanism may involve inhibition of phospholipase A_2 activity.

Key words: Glucocorticoid — Tumor necrosis factor — Cytotoxicity — Quinacrine

The tumor necrosis factor (TNF) was first discovered by Carswell *et al.* as a substance that induces necrosis of transplanted tumors *in vivo* and has cytotoxic effects on various tumors *in vitro*.[1] Recently, Large-scale production of human TNF was achieved by gene technology,[2,3] and methods for endogenous production of TNF have also been developed with a view to its application to cancer patients.[4–6] Based on these studies, the clinical use of TNF is now being investigated in various parts of the world.

TNF shows strong antitumor activity against solid tumors, especially tumors growing in skin tissues, but has little effect on tumor cells in ascitic fluids or blood when administered systemically. This difference in susceptibility of tumors of different histological types was suggested to be due to differences in their blood circulation and immunological responses.[7,8] From another point of view, we thought that the difference in susceptibility might be due to the presence of some intrinsic inhibitors of TNF activity in body fluids or cells.

Recently, Müller *et al.*[9] reported that the murine gene that codes TNF is mapped in a restricted region between H2-K and H2-D on chromosome 17. Two enzymes related to glucocorticoid metabolism (21-hydroxylases A and B) are also mapped in this region. Glucocorticoids are known to inhibit various types of immune responses and the production of cytokines such as interleukin 1, interleukin 2 and TNF.[10] These facts prompted us to examine whether glucocorticoid may be a natural intrinsic inhibitor of TNF activity or not.

Here we report that the cytotoxic activity of TNF against L929 cells is inhibited by very low, but physiological, concentrations of glucocorticoids as well as by a phospholipase A_2 inhibitor, quinacrine.

Purified recombinant human TNF[3] (specific activity 2.4×10^6 U/mg) was kindly supplied by Asahi Chemical Industry (Tokyo). Hydrocortisone, corticosterone, dexamethasone and quinacrine were purchased from Sigma Chemical Co., St Louis, USA. Cholesterol, progesterone and 17-OH progesterone were from Wako Pure Chemical Industry (Osaka). *In vitro* cytotoxicity against L929 cells was assayed by the methods of Ruff and Gifford,[11] as described in detail elsewhere.[4,12] Briefly, cultures of 8×10^4 cells in 0.1 ml of Eagle's minimum essential medium with 5% FCS were established in 96-well flat-bottomed microtiter plates and incubated in a humidified atmosphere of 5% CO_2 in air at 37° for 3 hr. Dilutions of test steroidal compounds in 20 μl of the medium were added to the wells, and the cells were incubated at 37° for 1 hr. Then dilutions of TNF in 80 μl of the medium were added, and after culture for

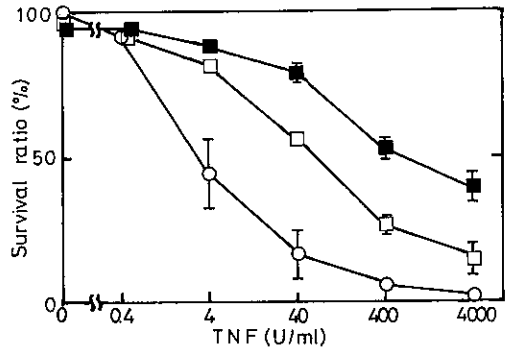

Fig. 1. Inhibition of TNF cytotoxicity by hydrocortisone. Cytotoxicity of TNF against L929 cells was tested in the absence of hydrocortisone (○) and in its presence at $1.0\times10^{-7}M$ (□) and $1.0\times10^{-6}M$ (■). Bars indicate deviations of values in duplicate samples when these were more than 2%.

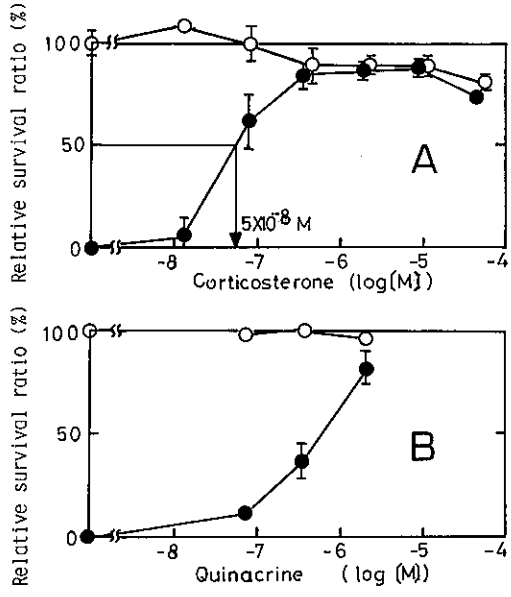

Fig. 2. Inhibitions of TNF cytotoxicity by corticosterone and quinacrine. The effects of corticosterone (A) and quinacrine (B) on the cytotoxicity of TNF (●, 40 U/ml) were tested. ○, without TNF. The relative survival ratio was calculated as follows: ([test sample] − [TNF 40 U/ml] ÷ (100 − [TNF 40 U/ml]) × 100. [ ] indicates the survival ratio. [TNF 40 U/ml] was 24% for Fig. 2A and 5% for Fig. 2B. The arrow represents the effective concentration of corticosterone. For details, see the text.

40–42 hr, the cells were examined by microscopy, and viable cells were stained with crystal violet. The absorbance at 550 or 590 nm was measured photometrically and the survival ratio was calculated from the formula: survival ratio = absorbance in test culture ÷ absorbance in TNF-free culture × 100 (%).

Figure 1 shows the dose-response curves to human TNF in the presence or absence of hydrocortisone. Without hydrocortisone in this system, the ED_{50} for cytotoxicity of TNF (50% survival ratio), was about 3 U/ml. In the presence of 10^{-7} and $10^{-6}M$ hydrocortisone, the ED_{50} values were 60 U/ml and 500 U/ml, respectively. Thus, hydrocortisone made L929 cells resistant to TNF and increased the ED_{50} of TNF more than 10-fold. Judging from the shape of the dose-response curve, the inhibiton was non-competitive.

The effects of other steroidal compounds on the cytotoxicity in the presence of 40 U/ml of TNF were examined. Of the compounds tested, corticosterone, which functions as a major glucocorticoid in mice, showed the strongest inhibitory activity. Figure 2 shows that the effective concentration of corticosterone for 50% inhibition of the cytotoxicity of TNF (40 U/ml) was $5\times10^{-8}M$.

Several precursors of these glucocorticoids, progesterone 17-hydroxyprogesterone and 11-deoxycorticosterone showed weak inhibitory effects, their effective concentrations being 2×10^{-6}, 6×10^{-6} and $1\times10^{-6}M$, respectively. Cholesterol (8×10^{-8}–$5\times10^{-5}M$) had no significant effect on the cytotoxicity. Dexamethasone, a synthetic steroidal anti-inflammatory agent, had a very strong inhibitory effect; its effective concentration was $5\times10^{-9}M$. These data indicate that glucocortitcoids with anti-inflammatory activity inhibit the cytotoxicity of TNF.

Glucocorticoids are known to inhibit inflammatory reactions through induction of *de novo* synthesis of lipocortin, a phospholipase A_2 inhibitor.[13,14] Using actinomycin D and cycloheximide, we examined whether *de novo* synthesis of RNA followed by protein is required for the inhibitory action of hydrocortisone on TNF cytotoxicity.

Actinomycin D and cycloheximide are reported to make target cells sensitive to TNF, shortening the culture period for the test of TNF cytotoxicity.[11,12,15] The data in

Table I. Effects of Actinomycin D and Cycloheximide on the Inhibitory Action of Hydrocortisone on TNF Cytotoxicity

Addition to culture	Hydrocortisone	[+TNE]/[−TNF][a] 20 hr culture	[+TNE]/[−TNF][a] 46 hr culture
—	−	45±3.6	2±2.1
	+	69±2.5	39±4.7
Actinomycin D	−	33±0.6	Not tested
	+	31±0.9	
Cycloheximide	−	21±1.1	Not tested
	+	16±2.9	

Actinomycin D (final concentration, 0.5 μg/ml), cycloheximide (4μM) or medium was added to culture wells just before addition of hydrocortisone 3 hr after seeding L929 cells, and TNF was added 1 hr later. Since the cells showed different sensitivities to TNF in the presence of these antimetabolites, the final concentrations of TNF in the wells were adjusted to 400 U/ml with medium, 0.004 U/ml with actinomycin D and 40 U/ml with cycloheximide.

a) Survival ratio of cells with TNF/that without TNF.

Table I confirm this and show that when L929 cells were cultured with TNF and/or hydrocortisone for 20 hr, hydrocortisone significantly inhibited the cytotoxicity of TNF in the absence of actinomycin D or cycloheximide. On the other hand, in the presence of actinomycin D or cycloheximide, hydrocortisone had no effect. This suggests that the inhibitory action of hydrocortisone on TNF cytotoxicity requires RNA and protein synthesis.

Thus, the inhibitory action of hydrocortisone on TNF-cytotoxicity may be ascribed to the *de novo* synthesis of lipocortin. If so, agents that inhibit phospholipase A_2 may be able to replace hydrocortisone in inhibiting TNF-activity. To test this possibility, we examined the effect of a phospholipase A_2 inhibitor, quinacrine, on the TNF cytotoxicity. As shown in Fig. 2B, $2\times10^{-6}M$ quinacrine clearly inhibited the TNF cytotoxicity.

This is the first report that glucocorticoids inhibit the biological activity of TNF. Normal human blood contains $1.3\text{–}5.5\times10^{-7}M$ (5–20 μg/dl) hydrocortisone, its concentration showing a circadian rhythm and also varying depending on other physiological conditions. Its concentration is very critical for the activity of TNF in the blood stream. As shown in Fig. 1, hydrocortisone at $1.0\times10^{-7}M$ clearly inhibited the cytotoxicity of TNF, suggesting that this concentration in the circulation is critical for resistance to TNF of tumor cells in tissues supplied with glucocorticoids by the blood stream.

The inhibition of TNF cytotoxicity by glucocorticoids apparently involves RNA and protein syntheses (Table I). We speculate that glucocorticoids may inhibit TNF cytotoxicity by induction of a lipocortin-like phospholipase A_2 inhibitor, because L929 cells can produce two types of lipocortins,[16] and the phospholipase inhibitor quinacrine inhibited the TNF cytotoxicity (Fig. 2B). The involvement of phospholipase A_2 in the cytotoxic activity if TNF is not surprising, because Kobayashi *et al.*[17] found that the cytotoxic action of lymphotoxin, which has structural and function homology to TNF, is mediated by activation of the phospholipase. This speculation does not exclude other possibilities, such as involvement of lysosome function in the mechanism of the inhibition of TNF cytotoxicity.

These findings explain the mechanism of the cytotoxicity of TNF, which has previously been unknown. For further clarification of the mechanisms regulating TNF cytotoxicity, the effects of TNF and glucocorticoids on lipid metabolism in the target cells must be examined.

Glucocorticoids regulate the cytotoxic activity of TNF, but they do not cause direct competitive inhibition of TNF (Fig. 1). Thus we speculate that glucocorticoids may regulate other biological activities of TNF such as induction of inflammatory reactions.[18,19] This idea is consistent with the anti-inflammatory action of glucocorticoids.

Finally we wish to note that, when TNF is administered to cancer patients, the critical level of glucocorticoids in their blood should be carefully monitored in relation to the antitumor effects, as well as side effects, of TNF and that antimetabolites of glucocorticoids should potentiate the action of TNF in cancer patients.

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