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VINBLASTINE-DEPENDENT DOWN-MODULATION OF TNF RECEPTORS IN HUMAN OSTEOSARCOMA CELLS IS MEDIATED BY PROTEIN KINASE C ACTIVITY

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The binding of tumor necrosis factor (TNF) to a human osteogenic sarcoma cell line (Saos-2) was investigated. These cells express two types of receptors as determined by specific monoclonal antibodies. Vinblastine induced a down-modulation of these receptors weaker than the one produced by phorbol esters or okadaic acid treatment. On exposure of cells to 10 μM vinblastine for two hours an approximately 55-65 % diminution of TNF binding was observed, but only 20% reduction occurred under long-term vinblastine treatment. TNF receptor down-modulation induced by vinblastine was partially prevented by protein kinase C inhibitors or protein kinase C depletion. It is suggested that the regulation of TNF binding to each one of its receptors in Saos-2 cells always occurs in a phosphorylation-dependent manner.

Tumor necrosis factor (TNF) (1,2), a potent cytokine secreted by activated macrophages, induces a variety of immune defence mechanisms. TNF is thought to play a critical role in mediation of the inflammatory response and resistance to infections and tumor growth. TNF interacts with a wide variety of cell types and upon binding to its receptors, it triggers a multiplicity of actions which include activation of signal transduction pathways, kinases and transcription factors, as well as induction of genes and proteins (reviewed in refs 3,4). This cytokine binds with high affinity to two distinct cell-surface receptors with molecular masses of 55-kDa (p55, TNF-RI) (5,6) and 75-kDa (p75, TNF-RII) (7,8). While the extracellular ligand-binding domains of these two receptors are similar, no homologies have been found in the intracellular portions (7,9) suggesting the possibility of the existence of distinct intracellular signalling pathways activated upon binding of the cytokine to each one of the receptors. Although the molecular mechanism of the selective binding of TNF to either the p55 or the p75 is still an unresolved problem, the preferential triggering by

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one of the two TNF-receptor complexes of a well-defined set of biological responses could account for the pleiotropic and often contradictory (beneficial or detrimental) nature of action of the TNF molecule (10).

Susceptibility of cells to TNF can be modulated by a variety of substances capable of affecting the affinity or the number of TNF receptors. They include protein kinase C activators or inhibitors (11-13), protein kinase A activators (13,14), and microtubule-disassembling agents (15). The lack of a consensus sequence typical of proteins phosphorylated on serine and threonine residues in the intracellular domain of the p55 and p75 (5,6,8), together with the effectiveness of microtubules depolymerizing agents in inhibiting the binding, has suggested that the effect of protein kinase C can be mediated by the phosphorylation of microtubule proteins (16).

The tumor promoter phorbol myristate acetate (PMA) and the protein-serine/threonine phosphatases (PP1 and PP2A) inhibitor okadaic acid are very potent inhibitors of binding of recombinant TNF to the human osteogenic sarcoma cell line Saos-2 as shown in a previous paper (17). Vinblastine and other microtubule disrupting agents, decreased the TNF binding only partially (50-65%). These data have suggested that the degree of binding of TNF to its receptors in Saos-2 cells is under the control of protein kinase C- and phosphatase-dependent, phosphorylation/ dephosphorylation systems, in association with the microtubule network (17).

In the present study we have found that the relative abundance of p55 and p75 receptors in Saos-2 cells is not affected by down-modulation produced by vinblastine or protein kinase C activation. We have also studied a situation in which vinblastine induced full microtubule disorganization, without substantial alteration of TNF binding to its receptors. The inhibitory action of vinblastine was also found to be prevented by specific protein kinase C inhibitors. We conclude that the regulation of TNF binding to its receptors is mainly realized by phosphorylation /dephosphorylation processes.

Materials and Methods

Cells and Reagents.

The cell line used, Saos-2 human osteosarcoma, was obtained from American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle medium containing 25 mM sodium bicarbonate, 60 U/ml penicillin, 60 µg/ml streptomycin, and 10% fetal calf serum. Cells were usually seeded into 6-multiwell plates and grown to confluence at 37°C in a humidified atmosphere of 5% CO_2. Growth media and serum for cell culture were obtained from Gibco Laboratories (Grand Island NY). Tumor necrosis factor (TNF human, recombinant) was from Boehringer Mannheim. Recombinant (^{125} I)-TNF (1.03 Ci/mmol) was from NEN Dupont. Vinblastine sulphate and phorbol myristate acetate (PMA) were from Sigma and staurosporine and calphostin C were supplied by LC Services Corporation (Woburn, MA). Ro-31-8830 (18) was generously provided by Dr. U. Moser (Hoffmann LaRoche, Basel). The anti-p55 (htr-9) and anti-p75 (utr-1) (19) monoclonal antibodies (mAbs) were a kind gift of Dr. W. Lesslauer, Hoffmann LaRoche, Basel.

Binding of (^{125}I)TNF to Intact Cells.

Confluent cultures of Saos-2 cells were employed for the binding assays. Cells were thoroughly washed with phosphate-buffered saline (PBS) and further incubated at 37°C with complete media in the presence of the indicated compounds. Then, cells were washed several times with buffer A (PBS/0.1% bovine serum albumin) and further incubated for one hour at 37°C with the indicated monoclonal antibodies. Afterwards, cells were washed with buffer A and incubated for additional two hours at 4°C with (^{125}I)TNF (6 ng/ml, 170,000 cpm) in buffer A. For determination of non-specific binding, a 250-fold excess of unlabelled TNF was added to the cultures. Usually non-specific binding was less than 15%. The incubation was terminated by extensively washes in buffer A, and then cells were solubilized in 0.1N NaOH/ 2% Na_2CO_3/ 1% sodium dodecylsulfate and radioactivity measured in a gamma counter.

Results

Saos-2 Cells Express the 75 kDa and 55 kDa TNF Receptors.

Preincubation of Saos-2 cells with a monoclonal anti-TNF receptor (p55) antibody, resulted in reduction of the TNF binding by approximately 80% as shown in Fig. 1. After preincubation of Saos-2 cells with the monoclonal anti-TNF receptor (p75) antibody, a 20% diminution of binding was seen. Combination of both antibodies totally inhibited TNF binding. Since both receptors are present in this cell line in a ratio 80/20, the next experiments were designed to answer the question whether the two receptors are equally regulated by vinblastine treatment.

Effect of Vinblastine on (^{125}I)TNF Binding.

Vinblastine, a microtubule-depolymerizing agent (15) induced in Saos-2 cells a reduction of (^{125}I)TNF binding in a dose dependent mode (17). However, vinblastine and other similar compounds like colchicine or vincristine never produced an inhibition bigger than about 50-65% of the maximal TNF binding, even at the maximal non-toxic concentration (15 μM) when incubated for two hours. The effect

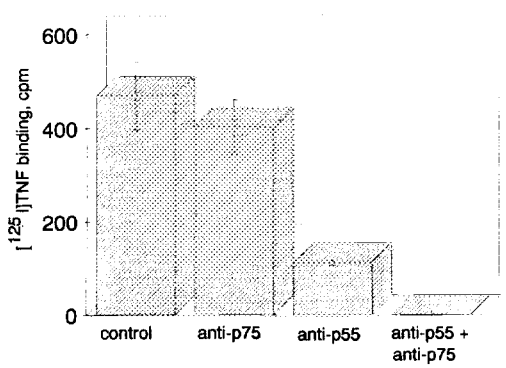

Figure 1. Effect of mAbs on the (^{125}I)-TNF binding to Saos-2 cells. Cells were incubated with the indicated mAbs (anti-p55, anti-p75 or a combination of both, final concentration 10μg/ml) at 37°C, washed and further incubated with (^{125}I)-TNF as described in "Materials and Methods". The values represent specific counts (after background subtraction) normalized for 5x10^5 cells.

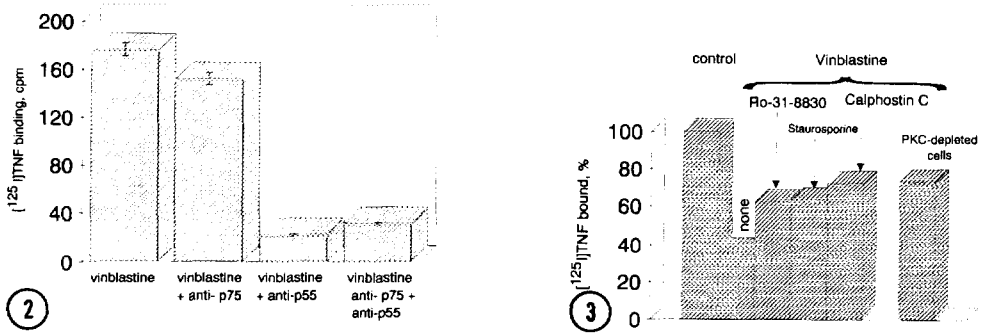

Figure 2. Effect of vinblastine on (^{125}I)-TNF binding. Cells were pretreated for 2 h with 10μM vinblastine, washed, incubated with the indicated mAbs for 1h at 37°C, followed by determination of (^{125}I)-TNF binding at 4°C as described in "Materials and Methods". The value of (^{125}I)-TNF bound to untreated cells was 580 cpm. The values represent specific counts (after background subtraction) normalized for $5x10^5$ cells.

Figure 3. Role of protein kinase C on the vinblastine-mediated down-modulation of TNF receptors. Cells were pretreated with the following PKC inhibitors: 1μg/ml Ro-318830, 15 nM staurosporine or 100 nM calphostin for 3h at 37°C. PKC-depleted cells were obtained by incubating them for 24h with 1μM PMA. Then, vinblastine (10 μM) was added to the cells and further incubated for 2h at 37°C, followed by (^{125}I) TNF binding determination. Bound ^{125}I label is expressed as percent of control cells without vinblastine.

of vinblastine was additive to that of PMA (17). The experiment of Fig. 2 shows that after vinblastine treatment the ratio of the receptors subpopulation is the same as obtained in its absence (80/20).Thus, it may be presumed that the inhibition by vinblastine of TNF binding affects only about the half of each receptor population.

It has been suggested that TNF receptors turn over very rapidly by internalization and that *de novo* protein synthesis is required for their replacement on the membrane. Incubation for 2 h with cycloheximide (10 μg/ml) decreased the TNF binding less than 20% (not shown) while the inhibition by vinblastine of TNF binding, during the same time, was more than 50 %. This indicates that, in osteosarcoma cells, the reduction of TNF binding caused by vinblastine was not due to inhibition of TNF receptor synthesis, as it may occur in macrophages and endothelial cells (15). To analyze the involvement of protein kinase C in the down-modulation induced by vinblastine, cells were treated with known inhibitors of the enzyme (calphostin C, staurosporine or Ro-31-8830). Under these conditions or in PKC-depleted cells it was observed a less pronounced inhibitory effect on TNF binding induced by vinblastine (Fig. 3), indicating a role of PKC on the vinblastine effects.

Maximal inhibition obtained with vinblastine was achieved in 2h incubation time. After 24 h treatment the level of TNF bound to Saos-2 cells reached similar values to control, untreated cells. Controls were performed to ensure that vinblastine was not destroyed during the 24-hours incubation time. The type of receptors present in Saos-2 cells after a long-term vinblastine treatment was again 20% and 80% for the p75 and p55 receptors, respectively (Fig. 4).

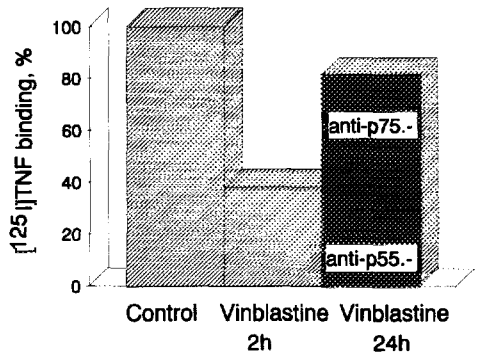

Figure 4. Comparison between the short and long-vinblastine treatment on the (^{125}I)-TNF binding and expression of p55 and p75 receptors. Cells were treated for 2 or 24 h with 10 μM vinblastine and further processed for TNF binding determination. After a 24h vinblastine pretreatment, cells were incubated with the indicated mAbs for 1h at 37°C, washed and TNF binding was measured as described before.
Results are expressed as percentage of control, untreated cells.

Discussion

Two species of TNF receptors exist, with similar ligand binding specificity and affinity but different effector portions. This suggests that regulation of this effector portion may be important in the selection of which information should be further elaborated and transferred into the cell. Therefore, the search of conditions and reactions able to identify and modulate putative regulatory pathways is important. The two receptor populations are both expressed in Saos-2 cells. To what extent the TNF binding is under the control of microtubule associated reactions and of phosphorylation events can be discussed on the basis of the presented experiments.

We have reported in this study, that vinblastine inhibits 50-65% of the TNF binding to its receptors despite the large amounts employed and full depolymerization of microtubules. The comparison of the time course of cycloheximide-induced inhibition of TNF binding and that of vinblastine suggests that the latter event is not the consequence of novel receptor synthesis and degradation.

Microtubule depolymerization has been described to cause a decrease in affinity of the TNF receptor with consequent TNF detachment. However conditions have been shown in which full depolymerization occurs (24 h vinblastine treatment) without a marked change in the binding of TNF compared to control cells. Thus, the two events appear not to be linked by a cause to effect relationship. Moreover, the effect of vinblastine is sensitive to protein kinase C inhibitors or protein kinase C depletion (Fig. 3) . In the presence or absence of vinblastine phorbol esters are able to produce an almost complete inhibition of TNF binding to its receptors. Since the TNF receptors lack sequences that can be phosphorylated by protein kinase C an alternative target for protein kinase C phosphorylation must be postulated. It is conceivable that a microtubule associated protein, if phosphorylated by PMA-activated protein kinase C, will dissociate from the microtubules and induces a diminution of the TNF receptor affinity for TNF. Okadaic acid inhibition of TNF binding mimics that of PMA and is

consistent with the inhibition of dephosphorylation of a protein kinase C target, without involving an additional mechanism of action (20). Depolymerization of microtubules may be effective in facilitating the phosphorylation of such a protein responsible for diminishing TNF binding. Microtubules depolymerization by vinblastine however does not appear to be essential as such in modulating TNF receptor affinity. The presence of two types of receptor may suggest that the p75 and p55 are regulated by different mechanisms. However, the use of specific antibodies against the two receptors suggests that in both cases receptor affinity is mainly regulated by protein kinase C. It can be thus concluded that depolymerization of microtubules is not always a prerequisite for the diminution of receptor affinity for TNF and that the effect of phorbol esters is not mediated by microtubule depolymerization.

Acknowledgments

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