DIFFERENTIAL CYTOSTATIC ACTIVITY OF MONOCYTE-DERIVED CYTOKINES AGAINST HUMAN MELANOMA CELLS

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We investigated the capacity of 3 major cytokines secreted by activated monocytes, IL-1β, TNFα and IL-6, to inhibit growth of melanoma tumor cells. Using neutralizing antibodies against IL-1β, TNFα and IL-6, we observed that the cytostatic activity against A375 melanoma cells is largely due to the presence of IL-6 in culture supernatants of monocytes stimulated with LPS. A375 cells appeared to be extremely sensitive to monocyte-derived cytokines, since in addition to rIL-1β and rTNFα, rIL-6 displayed cytostatic activity against A375 cells. We observed additive or synergistic cytostatic effects upon use of combinations of these cytokines. When 7 other melanoma cell lines and short-term melanoma cultures were tested and compared with A375, a major difference in their sensitivity to monocyte-derived cytokines was observed. Although 7 out of 8 melanoma cell lines were sensitive to culture supernatants of monocytes stimulated with LPS, significant differences were found when recombinant cytokines were used. The widely used A375 was the only melanoma cell line sensitive to rIL-1β, rTNFα and rIL-6. The growth of none of the other 7 melanoma cell cultures was significantly affected by rIL-1β. Seven out of 8 melanoma cell cultures were sensitive to rTNFα and 3 out of 8 to rIL-6. The results of our study indicate that the sensitivity of melanoma cell cultures for different monocyte-derived cytokines is highly variable, and that it is questionable whether the A375 melanoma cell line, sensitive to rIL-1β, rTNFα and rIL-6, is representative for melanoma.

Monocytes activated by LPS produce a number of cytokines with cytostatic activity for certain tumor cells. Dependent on the target cells used to measure anti-tumor cytostatic/cytotoxic activity of monocytes, various studies report that this effect must be ascribed to IL-1β and TNFα secreted by monocytes (Onozaki et al., 1985; Ruggiero and Baglioni, 1987). If human A375 melanoma cells are used as target cells, both monokines, either alone or in combination, have been shown to possess potential anti-tumor effects (Ruggiero and Baglioni, 1987; Ichinose et al., 1988; Nakai et al., 1988). However, with the availability of recombinant cytokines, data are accumulating that many of the activities that were formerly ascribed to purified IL-1 preparations may be mediated by other contaminating cytokines present in the purified material (Wong and Clark, 1988; Helle et al., 1988b). Furthermore, the biological assays by which the IL-1 activity was determined were also found to be sensitive for TNFα and IL-6 (Wong and Clark, 1988; Van Damme et al., 1988). In addition, IL-1 can induce the production of IL-6 in monocytes (Tosato and Jones, 1990) and IL-1 and TNFα can induce each other's release by monocytes (Dinarello et al., 1986). These observations prompted us to investigate the cytostatic activity of monocyte-derived IL-6.

Our results demonstrate that not IL-1β and/or TNFα but IL-6 is the major cytokine with cytostatic activity against A375 melanoma cells in monocyte culture supernatant. However, the sensitivity of different melanoma cell lines to these 3 cytokines was highly variable.

MATERIAL AND METHODS

Monocytes and monocyte culture

Human peripheral-blood monocytes were isolated and cultured as described by Figdor et al. (1982) and Te Velde et al. (1988). Briefly, mononuclear cells were separated by density centrifugation with a blood-component separator, followed by fractionation into lymphocytes and monocytes by centrifugal elutriation. Monocytes (≥95% pure) were cultured in Yssel's medium (Yssel et al., 1984) containing human serum albumin and supplemented with 1% heat-inactivated human serum and 1 μg/ml of LPS in a concentration of 4.10¹⁶ cells per ml at 37°C, 5% CO₂ and 100% humidity in Teflon bags (Jansen MNL, St. Niklaas, Belgium). After culture for 4 hr, the culture supernatant was harvested and stored at −20°C until required.

Melanoma cell lines

The melanoma cell lines are: A375 (Giardi et al., 1973), M14 (Katano et al., 1984), MeWo (Carey et al., 1976), Mel 518 A2 (Versteeg et al., 1988) and BRO (Lockshin et al., 1985). The short-term-cultured (<2 months in culture) HR, ZR and KZ were established from metastatic melanoma lesions in our laboratory. The melanoma cells were cultured in Iscove's medium supplemented with 10% heat-inactivated FCS, 0.2 mM glutamin and antibiotics. The melanoma cell lines were free of Mycoplasma contamination.

Recombinant cytokines and neutralizing antibodies

Recombinant human IL-1β (1,000 U/ml) was purchased from Boehringer, Mannheim, Germany. Human rTNFα (specific activity 6.10⁷ U/ml) was provided by Boehringer Ingelheim, Vienna, Austria. Human rIL-6 (specific activity 10⁶ U/mg) and neutralizing anti-IL-6 antibodies were kindly provided by Drs. M. Helle and L.A. Aarden (Brakenhoff et al., 1987) from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands. Human rGM-CSF (cos 7 culture supernatant) was a generous gift from Drs. P. Trotta and S. Nagabhushan (Schering-Plough, Bloomfield, NY). Neutralizing mouse anti-IL-1β monoclonal antibodies (MAbs) F1B1 and F1B3 were kindly provided by Dr. H. Herzbeck (Forschungsinstitut Borstel, Germany). Neutralizing mouse anti-TNFα MAbs were a generous gift from Dr. R. Torensma (University of Utrecht, Utrecht, The Netherlands). Non-specific purified mouse anti-idiotypic MAbs (K8) and normal goat serum were used as control antibodies.

Detection of IL-1α, IL-1β, TNFα and IL-6 in monocyte culture supernatant

IL-1α in the culture supernatant of human monocytes was tested using the IL-1α ELISA (Endogen, Boston, MA). The sensitivity of this assay is 50 pg/ml of IL-1α and there is no cross-reactivity with IL-1β, IL-2, IFNs, IL-6, TNFα, lymphotoxin or other cytokines.

IL-1β in the culture supernatant of human monocytes was tested using the IL-1β ELISA assay (Cistron, Pine Brook, NJ). The sensitivity of this assay is 20 pg of IL-1β and there is no cross-reactivity with IL-1α, IL-2 or TNFα.
TNFα in the culture supernatant of human monocytes was tested using the Biokine TNF Test Kit (T-cell Sciences, Cambridge, MA). The sensitivity of this assay is 10 pg TNFα/ml and no detectable cross-reactivity with other described cytokines can be observed.

IL-6 in the culture supernatant of human monocytes was tested using the R and D Systems Quantikine human IL-6 immunoassay (Minneapolis, MN). The sensitivity of this assay is 3.5 pg/ml and it shows no detectable cross-reactivity with other known cytokines.

**Growth inhibition assay**

Melanoma cells were seeded (1,000/well of a 96-well plate) in Iscove’s medium supplemented with 10% heat-inactivated FCS in a volume of 100 μl. After 72 hr of incubation at 37°C and 5% CO2 the cells were pulsed for 4 hr with 0.4 μCi/mmol (NEN, Boston, MA), after which the cells were harvested and counted. The data are expressed as percentage growth inhibition calculated by the formula:

\[
\text{growth inhibition} = \left(1 - \frac{\text{cpm test sample}}{\text{cpm medium (no additives)}}\right) \times 100\%
\]

<table>
<thead>
<tr>
<th>Monokine</th>
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<td>330 (60-830)</td>
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<tr>
<td>TNFα</td>
<td>20.5 (0.5-44.5)</td>
<td>340 (10-740)</td>
<td>5</td>
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<tr>
<td>IL-6</td>
<td>22.0 (3.0-30.0)</td>
<td>14,700 (2000-19,500)</td>
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**RESULTS**

Blocking of cytostatic activity of monocyte culture supernatant by neutralizing antibodies

We have shown that monocyte culture supernatant contained factors with cytostatic activity for A375 cells (Te Velde et al., 1988). Monocytes activated by LPS rapidly secrete IL-1β, TNFα and IL-6. The concentration of the secreted monokines varies between monocyte cultures from different donors as shown in Table I. To investigate whether IL-1β, TNFα and/or IL-6 were responsible for the cytostatic activity found in culture supernatant of LPS-activated monocytes, we performed blocking experiments with antibodies in saturating concentrations that neutralized cytokine activity. Figure 1a shows a dose-response curve of the growth-inhibitory effect of monocyte culture supernatant on the melanoma cell line A375. The results indicate that 5% culture supernatant causes maximal growth inhibition. MAbs against IL-1β did not neutralize the cytostatic activity present in high concentrations in the monocyte supernatant (Fig. 1b). Also anti-TNFα MAbs alone did not cause significant reduction of maximal cytostatic activity. Since IL-1β and TNFα can act synergistically on several tumor cell lines (Ruggiero and Baglioni, 1987), we studied the effect of combinations of anti-IL-1β MAbs with anti-TNFα MAbs (Fig. 1c). However, no additional blocking of the cytostatic activity by either anti-IL-1β or anti-TNFα was observed. Only at dilutions of the monocyte supernatant of 1:100 to 1:500 could a 50% reduction of the inhibition of cytostatic activity be observed with anti-IL-1β and/or anti-TNFα antibodies (Fig. 1, b, c). These data indicate that the cytostatic activity is not mediated via IL-1β and/or TNFα and must be ascribed to other cytokines produced by monocytes after LPS stimulation. Because IL-1 and IL-6 have a number of overlapping activities (Wong and Clark, 1988) and since IL-1

![Figure 1](image-url)

**Figure 1** – Blocking of the cytostatic activity present in monocyte culture supernatant with neutralizing antibodies directed against cytokines. The monocyte culture supernatant contained average concentrations of the different cytokines: 7 ng/ml of IL-1β, 14 ng/ml of TNFα and 25 ng of IL-6. Growth inhibition of A375 melanoma cells was determined as described in the text. The following antibodies were tested: control mouse MAAb K8 (for anti-IL-1β and anti-TNFα), control NGS (for anti-IL-6), anti-IL-1β, anti-TNFα and/or anti-IL-6. The anti-IL-1β antibodies were used in a concentration of 100 μg/ml and the anti-TNFα and anti-IL-6 antibodies were used in a dilution of 1/100. These antibody concentrations neutralized 100 U of rIL-1β, 10,000 U of rTNFα and 10,000 U of rIL-6 for 75, 83 and 95%, respectively. Results of 1 representative experiment out of 3.
can induce IL-6 activity (Tosato and Jones, 1990), we tested the effect of anti-IL-6 antibodies on the cytostatic capacity of LPS-activated monocyte culture supernatants. As shown in Figure 1d, addition of anti-IL-6 antibodies almost completely abrogates the growth-inhibitory capacity of the monocyte supernatant. Combination of anti-IL-6 MAb with anti-IL-1β MAb and/or anti-TNFα MAb did not cause additional effects (Fig. 1e, f). Anti-IL-1β and/or anti-TNFα cause a 50% reduction of the cytostatic activity in the presence of low concentrations of IL-6, indicating that IL-1β and/or TNFα have synergistic or additive effects on the cytostatic activity at low concentrations of IL-6.

Taken together, our results indicate that IL-1β and/or TNFα are not the factors in activated monocyte supernatants that are responsible for cytostatic effects against human A375 cells, but that IL-6 appears to be the major cytokine with cytostatic activity against A375 cells produced by activated monocytes.

The finding that IL-6 is the major cytokine with cytostatic activity in monocyte culture supernatant does not exclude IL-1β and/or TNFα possessing cytostatic activity. To investigate whether IL-1β and/or TNFα have cytostatic activity against the human melanoma cell line A375, we performed a growth-inhibition assay with recombinant cytokines. The results (Fig. 2) demonstrate that rIL-6 as well as rIL-1β and rTNFα have a cytostatic effect on A375 cells in a dose-dependent fashion. Biologically effective concentrations of another monocyte-derived cytokine (rGM-CSF) did not display cytostatic activity. Purified recombinant forms of IL-1β and TNFα display marked cytostatic activity, but the concentrations needed to measure this exceed the concentrations present in monocyte culture supernatants. To exclude the possibility that the monocyte supernatants contain inhibitors of monokine activity, recombinant cytokines were added to the monocyte culture supernatants. No inhibition of the activity of the recombinant cytokines was found (data not shown), suggesting that the inability of IL-1β and TNFα to mediate cytostatic activity was not caused by secretion of inhibitory factors by the monocytes. To show that the lack of neutralizing capacity of anti-IL-1β MAb and anti-TNFα MAb was not caused by a general inability of the antibodies to neutralize cytokine activity, we performed blocking experiments with purified recombinant cytokines. We show that anti-IL-1β MAb as well as anti-TNFα and anti-IL-6 MAbs specifically block the respective cytostatic effects of the recombinant cytokines on A375 melanoma cells (results not shown).

In conclusion, our data indicate that IL-6 is the main factor in mediating monocyte cytostatic activity against A375 melanoma cells.

Additive and synergistic effects of rIL-1β, rTNFα and rIL-6

The data shown in Figure 1 (b and c) suggest that low concentrations of IL-1β, TNFα and IL-6, IL-1β and/or TNFα present in diluted monocyte supernatants can exert synergistic or additive effects with IL-6 on the growth of the A375 melanoma cell line. Therefore we investigated mixtures of low doses of rIL-1β and rTNFα, rIL-1β and rIL-6, and rTNFα and...
rIL-6 in a growth-inhibition assay using A375 cells. Additive or sometimes synergistic effects can be observed if rIL-1β and rTNFα are combined (Fig. 3a). However, much higher antitumor activity was observed when IL-1β and IL-6 were combined (Fig. 3b). True synergistic effects were measured when IL-6 was combined with low concentrations of TNFα (Fig. 3c). Concentrations of IL-1β and TNFα, which by themselves have low cytostatic activity on A375 cells, are effective if combined with low concentrations of IL-6. These results indicate that low concentrations of IL-1β, TNFα and IL-6 administered together have additive or synergistic effects on the growth of A375 cells.

**Effect of IL-1β, TNFα and IL-6 on melanoma cells other than A375**

A375 melanoma cells are extremely sensitive to monocyte-derived cytostatic factors and this cell line is the most commonly used target in these assays (Ruggiero and Baglioni, 1987; Ichinose *et al.*, 1988; Nakai *et al.*, 1988). We were interested to determine whether other melanoma cell lines were sensitive to monocyte-derived cytokines, and especially if these cells were also sensitive to IL-6. The cytostatic effect of monocyte supernatant (after 4 hr incubation with LPS) was compared with that of recombinant IL-1β, TNFα, IL-6 and GM-CSF and tested on 4 different melanoma cell lines (Fig. 4). A375 and M14 are equally sensitive to cytostatic factors present in the monocyte culture supernatant, followed by Mel 518 A2 and MeWo, which appear to be insensitive to factors present in this supernatant. Exposure of the melanoma cells to the different recombinant monokines showed that only A375 cells are sensitive to rIL-1β, whereas A375, M14 and Mel 518 A2 are sensitive to rTNFα (Fig. 4). The growth of MeWo, which is not sensitive to monocyte-supernatant-derived factors, is affected neither by rTNFα nor by IL-1β. IL-6 affects the growth of both A375 and M14, although the latter is less sensitive. MeWo and Mel 518 A2 are relatively insensitive to rIL-6. rGM-CSF has no significant effect on the growth of any of the melanoma cell lines tested (Fig. 4). Table II shows the comparison of the A375 melanoma cell line with a cell line derived from a primary melanoma lesion and 3 short-term cultures (< 2-month culture) of a metastatic melanoma lesion. Despite the low proliferative capacity exhibited by the short-term cultures, we found that these cultures also display variability in sensitivity to cytokines.

From these data we conclude that, as illustrated by this randomly chosen panel of melanoma cell lines and short-term cultures, the sensitivity of melanoma cells for monocyte-derived factors is highly variable.

**DISCUSSION**

The monocyte-mediated cytostatic activity against A375 melanoma cells can be ascribed to IL-6. Both rIL-1β and rTNFα display cytostatic activity against A375 cells, but compared with IL-6 the contribution of these monokines in monocyte supernatants to inhibit cell growth is limited. These results are in accordance with the data of a number of groups who describe similar cytostatic activity of rIL-1β and rTNFα on various tumor cell lines (Ruggiero and Baglioni, 1987; Ichinose *et al.*, 1988; Nakai *et al.*, 1988; Mortarini *et al.*, 1990) which can be blocked using specific anti-sera (Ichinose *et al.*, 1988; Nakai *et al.*, 1988). However, several reports show cytotoxic/cytostatic activity of IL-1 using a natural purified form derived from monocyte supernatants (Onozaki *et al.*, 1985; Lachman *et al.*, 1986). The biologic activity of these IL-1 preparations was determined with the murine thymocyte co-stimulatory assay. This assay is not specific for IL-1, since TNFα (Hurme, 1988) and IL-6 (Van Damme *et al.*, 1988) also contribute to thymocyte proliferation. Indeed, some of the purified natural IL-1 preparations were shown to be contaminated with IL-6 (Helle *et al.*, 1988). Moreover, anti-IL-1 anti-sera raised against monocyte culture medium contained anti-IL-6 activity (Aarden *et al.*, 1987). It can therefore not be excluded that at least
part of the reported cytostatic activity ascribed to IL-1 must be attributed to TNFα and IL-6.

When the contribution of different cytokatic factors present in monocyte supernatant was compared with single and combined recombinant factors a discrepancy was observed (Figs. 1–3). In principle, cytokines present in monocyte culture supernatant should not behave differently from heterologous cytokines. However, the ultimate growth-inhibitory capacity of a monocyte culture supernatant is an end result of both inhibitory and stimulatory activities. The inability of anti-IL-1β and anti-TNFα to block monocyte-mediated growth inhibition could not be ascribed to IL-1β and TNFα inactivity caused by inhibitors present in the culture medium, inasmuch as addition of rIL-1β or rTNFα to the monocyte culture supernatant inhibited tumor growth (results not shown). A possible explanation for this finding is that, compared with IL-1β and TNFα, monocytes produce much higher levels of IL-6 activity. In Table I it was shown that 5% monocyte supernatant causing maximal growth inhibition (Fig. 1) contains approximately 16.5 U of IL-1β, 17 U of TNFα and 735 U of IL-6. However, since the units for IL-1β, TNFα and IL-6 are determined in different assays, a direct comparison of relative activity based on these units is not justified. Moreover, factors as yet unidentified may influence the result obtained with monocyte culture supernatant.

The exact mechanism of regulation of monokine production remains largely unknown, the more so as IL-1, TNFα and IL-6 can influence each other’s release (Dinarello, 1989). In addition, not only monocytes but also melanoma cells produce IL-1 and IL-6. Melanoma cells have the capacity to produce IL-1α (Köck et al., 1989; Benincelli et al., 1989) and Morinaga et al. (1989) have demonstrated that IL-1-sensitive melanoma cells can produce IL-6 upon treatment with IL-1α, the elaborated IL-6 acting as an autocrine inhibitor of cell proliferation. However, IL-6 production was moderate and resulted in a growth inhibition of less than 30%. The finding that melanoma cells secrete IL-1α and that IL-1α is involved in stimulation of melanoma cell IL-6 production suggests that regulation of cytokine secretion is complex. An important point that requires further investigation is whether melanoma cells also produce cytokines in vivo or whether the observed capacity to secrete cytokines is acquired by in vitro culturing.

Compared with 3 other melanoma cell lines which are relatively insensitive to one or more of the monocyte-derived cytokines, the A375 melanoma cell line is unique, since it is sensitive to rIL-1β, rTNFα and rIL-6 (Fig. 4). This cell line is therefore unsuitable for measuring IL-1 activity in a bioassay, as suggested by Nakai et al. (1988), since effects of TNFα and IL-6 cannot be excluded. The observed sensitivity for IL-6 appears to be confined to only a few melanoma cell lines: only 3 out of 8 melanoma cultures are more or less sensitive for IL-6. So it is questionable whether the A375 cell line is representative for melanoma in general.

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