Human Monocytes Both Enhance and Inhibit the Growth of Human Pancreatic Cancer in SCID Mice

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Abstract. *Background: Monocytes/macrophages exhibit antitumour potential, but clinicopathological evidence suggests that they may both inhibit and enhance tumour growth. The purpose of this study was to determine the effect of monocytes on the growth of human pancreatic cancer (HPC-4) in severe combined immunodeficient (SCID) mice. Materials and Methods: Freshly isolated human monocytes or $CD14^+$ cells from cocultures with tumour cells were coengrafted, at various ratios, with HPC-4 cells into SCID mice. The tumour size and angiogenesis were determined. Results: At a high ratio of monocytes to cancer cells the enhancement and, at a low ratio, the inhibition of tumour growth was observed. Multiple intratumoral applications of monocytes in large numbers also enhanced tumour growth. Deactivation of monocytes by a short pre-exposure to tumour cells in vitro before engraftment led to increased tumour growth. Monocytes used in large numbers and deactivated monocytes in low doses enhanced tumour-induced angiogenesis. Conclusion: Monocytes may both facilitate and suppress the growth of human tumours in SCID mice and both the number of monocytes, as well as the state of monocyte deactivation are critical for the final outcome of monocyte-tumour interactions.*

Tumour infiltrating macrophages (TIM) originate from blood monocytes recruited to the tumour site. Although both monocytes and macrophages have potential cytotoxic activity against tumour cells and produce several toxic mediators, such as tumour necrosis factor α (TNF), reactive oxygen (ROI) or nitrogen (RNI) intermediates they also produce protumour mediators, such as proangiogenic molecules. Clinicopathological evidence has indicated that TIM may both inhibit and enhance tumour growth (1). The evidence for these opposing effects of TIM has been derived from observations that the level of TIM infiltration correlated with the prognosis of invasive breast, colorectal and pulmonary cancer (2, 3, 4) and the function of TIM in comparison to blood monocytes was depressed in cancer patients (5). Other results have shown that TIM express the M2 phenotype which is associated with proangiogenic activity and supression of adaptive immunity (6, 7). Our *in vitro* data have indicated that repeated contact of monocytes with cancer cells resulted in diminished production of cytotoxic molecules (TNF, ROI) and immunomodulatory interleukin (IL)-12, and enhanced production of immunosupressive IL-10 (8). IL-10, which inhibits nuclear factor-κB (NF-κB) (9) is responsible for suppression of IL-12 production which is essential for the induction of cell-mediated immunity (10). The prompt assessment of the role of macrophages in human cancer is important as they are potential cellular targets of cancer immunotherapy (11).

This study was designed to determine the effect of monocytes on the growth of human pancreatic cancer in severe combined immunodeficient (SCID) mice and on angiogenesis.

Materials and Methods

Animals. SCID (CB17/Icr-Prkdc/Crl) mice were purchased from the Charles River Laboratories (Sulzfeld, Germany) and bred in the local animal facilities under pathogen-free conditions. Only of one sex, 8-12 weeks old, mice were used in the experiments. There were 4-6 mice per group. Ten to twelve weeks old Balb/c mice, bred in the local animal facility, were used for determination of angiogenesis.

Tumour cells. The human pancreatic adenocarcinoma (HPC-4) cell line isolated in our laboratory was used (12). The cells were cultured by biweekly passages in RPMI 1640 (Biochrom, Berlin, Germany) medium supplemented with gentamycin (50 μg/ml, Biochrom), glutamine (2 mM, Gibco, Paisley, UK) and 5% fetal calf serum (FCS, Biochrom), further referred to as complete

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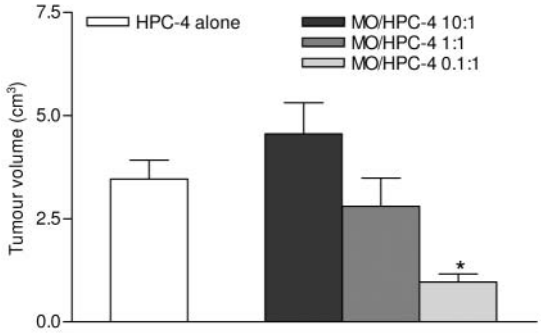

Figure 1. *The effect of monocytes (MO) on HPC-4 tumour growth. Different numbers of MO were mixed with HPC-4 cancer cells ($1x10^6$) and coengrafted into SCID mice. As control HPC-4 cells injected alone were used. Tumour size was measured at 63 days. Results are expressed as mean±SE of 5 different experiments. *Significantly different ($p<0.05$) in comparison with control.*

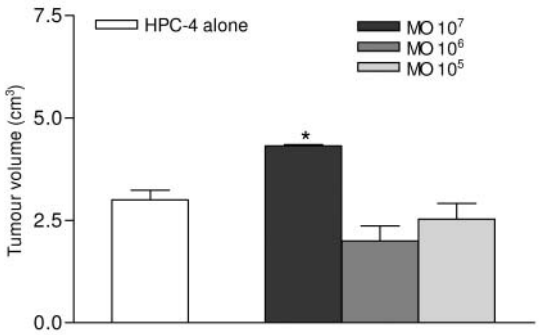

Figure 2. *The effect of multiple intratumoural injections of monocytes (MO) on the growth of HPC-4 tumours in SCID mice. MO ($1x10^7$, $1x10^6$, $1x10^5$) were injected into the growing tumour, once a week. Five injections of MO were given. Results are expressed as mean±SE of 5 different experiments. *Significantly different ($p<0.05$) in comparison with control.*

medium. The HPC-4 cells were regularly tested for Mycoplasma sp. contamination by Mycoplasma PCR ELISA test (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's protocol.

Isolation of cell populations. Human peripheral blood mononuclear cells (PBMC) were isolated from EDTA-blood of healthy donors by the standard Ficoll/Isopaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. The monocytes were separated from the mononuclear cells by counter-flow centrifugal elutriation with a JE-5.0 elutriation system equipped with a 5 ml Sanderson separation chamber (Beckman, Palo Alto, CA, USA) as previously described (13). The isolated monocytes were 90-96% pure, as judged by flow cytometry analysis (FACSCalibur, Becton Dickinson Immunocytometry System, San Jose, CA, USA) using fluorescein isothiocyanate (FITC)-conjugated anti-CD14 (Leu-M3, Becton Dickinson Biosciences, San Diego, CA, USA) monoclonal antibody (mAb). The cells were suspended in PBS. In some experiments monocytes isolated from coculture with tumour cells (preexposure) at the ratio 1:0.3 for 3 h, were used. After staining with phycoerythrin (PE)-labelled anti-CD14 (BD Biosciences) mAb, $CD14^+$ cells were isolated by sorting in a FACS Ventage Cytometer (Becton Dickinson Immunocytometry System) equipped with a Power Macintosh 7600/120 computer using Cell Quest v. 3.0 software (14). The purity of the sorted cells was checked by flow cytometry and exceeded 95%. As control, $CD14^+$ cells were sorted out from monocytes cultured in medium alone ("dummy sorting").

Tumour cell-monocyte engraftment. HPC-4 ($1x10^6$) cancer cells were mixed with monocytes, isolated from the blood or from cocultures with HPC-4 cells, at 1:10 - 1:0.1 ratios in a total volume of 0.2 ml of PBS and injected subcutaneously (*s.c.*) into the dorsal middle line area of 10-12 week-old SCID mice. The tumour size was recorded at 63 days. The longest dimension (a) and perpendicular width (b) were measured with a caliper and the tumour volume (v) was calculated according to the formula: $v = (ab)^2/2$ (15). In some experiments different numbers of monocytes ($1x10^7$, $1x10^6$, $1x10^5$) were injected intratumourally (once a week) five times when the tumour reached measurable size (usually around the 28th day).

Evaluation of cytokine production in vitro. HPC-4 cells ($5x10^5$/ml) and monocytes at 1:10, 1 or 0.1 ratios were cultured in the complete medium for 24 h. Then the supernatants were harvested and the concentration of cytokines was measured by appropriate ELISA kit: TNF, IL-10 (BD Biosciences), transforming growth factor (TGF)β (R&D Systems Inc., Abingdon, UK), IL-1β (Genzyme Diagnostics, Cambridge, MA, USA) and IL-18 (MBL Co. Ltd., Nagoya, Japan) according to the manufacturers' instructions.

Matrigel angiogenesis assay. HPC-4 cancer cells ($1x10^6$) mixed with monocytes or sorted $CD14^+$ cells (at different ratios) in 100 μl of PBS were added to 400 μl of Matrigel Matrix (BD Biosciences, Bedford, MA, USA) and injected *s.c.* into the abdominal middle line of Balb/c mice. HPC-4 cancer cells, monocytes and $CD14^+$ cells alone were used as controls. After 6 days the mice were euthanized, the Matrigel Matrix pellets were cut off, 400 μl of BD Cell Recovery Solution (BD Biosciences) was added and left at 4˚C for 7 days to release the cells. Then, the Matrigel Matrix implants were suspended in Drabkin solution (Sigma, St. Louis, MO, USA) and after 30 min spun down and the content of haemoglobin was determined by measuring absorbance at 540 nm in a U-1800 spectrophotometer (Hitachi, Tokyo, Japan) (16).

Results

The enhancement and inhibition of tumour growth by monocytes. Monocytes coengrafted with HPC-4 cells at the ratio 10:1 led to a notable enhancement of tumour growth (Figure 1). Some inhibition of the tumour growth was observed when monocytes were used at the ratio 5:1 (not shown). Paradoxically, low number of monocytes (ratio 0.1:1) caused a significant inhibition of the tumour development.

Multiple intratumoral applications of monocytes and tumour growth. In these experiments, after the appearance of measurable tumour, monocytes were injected 5 times, once a week, beginning at 1 month after tumour cell

Table I. *Cytokine production (pg/ml) by monocytes cocultured with cancer cells at different ratios.*

HPC-4/monocytes	IL-1β	IL-6	IL-10	IL-18	TNF	TGFβ
1:10	432±108	7249±2344	5539±947	33±19	1252±190	581±35
1:1	340±91	6178±920	510±170	37±23	1446±569	533±29
1:0.1	107±47	698±223	58±37	6±2	246±128	358±170
HPC-4 $1x10^6$	ND	ND	160±76	ND	ND	560±103
Monocytes $1x10^7$	ND	ND	355±37	ND	ND	154±65

ND: not detected.

engraftment. Enhancement of tumour growth following injection of a large number (10^7 per dose) of monocytes was seen (Figure 2). Smaller numbers of monocytes (10^6 and 10^5 per dose) caused nonsignificant inhibition of tumour growth but multiple injections were less effective than single ones, suggesting that an additional supply of monocytes to the tumour site is of a little importance for control of tumour growth.

Analysis of cytokine production in vitro by monocytes stimulated with tumour cells. In order to establish whether different levels of pro- and anti-inflammatory cytokines may be associated with the enhancing or inhibitory effects of the monocytes on tumour growth *in vivo*, the monocytes were cultured with cancer cells in the same ratios as used for engraftment into the SCID mice. No significant differences in the levels of IL-1β, IL-18, TNF and TGFβ were observed in these cultures (Table I). At the highest monocyte/tumour ratio the level of IL-6 in comparison to the lowest ratio was increased approximately 10 times and IL-10 approximately 90 times. Of note, the HPC-4 cells and monocytes at high numbers cultured alone also produced IL-10.

Preexposure of monocytes to tumour cells in vitro and their tumour promoting effect in vivo. Some of our previous observations indicated that the contact of monocytes with cancer cells induced an antitumour response of the former as measured by production of cytokines, ROI and cytotoxic/cytostatic activity (13, 14). Therefore, we assumed that tumour cell-preexposed monocytes should be more effective in the inhibition of tumour growth. Monocytes were cultured with tumour cells at the ratio 8:1 for 3 h and then the mixture of cells was injected into SCID mice. HPC-4 cells kept in medium alone served as control. The tumour cell-preexposed monocytes showed a higher tumour enhancing effect as compared to the control monocytes (Figure 3). In order to clarify the mechanism, $CD14^+$ cells were isolated from the cocultures with HPC-4 cells or "dummy sorted" (control), and then injected into SCID mice with tumour cells at 1:1 and 0.1:1 ratios. As shown in Figure 4, $CD14^+$ cells

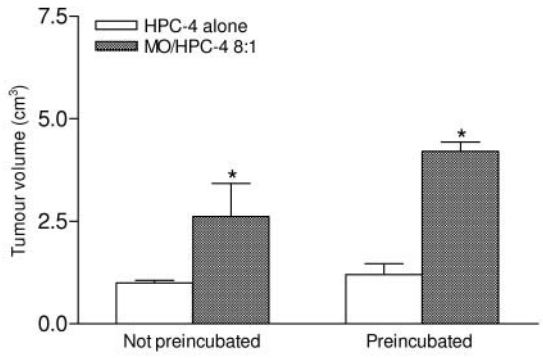

Figure 3. *The effect of in vitro preincubation of monocytes (MO) with HPC-4 cells on the growth of tumours in SCID mice. MO were cultured with tumour cells or in medium alone (control) for 3 h and, after washing, the cells were engrafted into SCID mice. Results are expressed as mean±SE of 5 different experiments. *Significantly different ($p<0.05$) in comparison with control.*

isolated from the *in vitro* coculture with HPC-4 cells, *i.e.* preexposed to tumour cells, significantly stimulated tumour growth even at the lower ratio, while the small number of $CD14^+$ control monocytes were inhibitory. Thus the preexposure of the monocytes to cancer cells dampened their antitumour effect.

The enhancement of tumour-induced angiogenesis by monocytes. Since several cytokines possess proangiogenic activity (17, 18) the ability of different numbers of human monocytes to induce angiogenesis in mice was assessed by Matrigel assay. Addition of a higher number of monocytes enhanced tumour cell-induced angiogenesis (Figure 5). Monocytes alone showed little angiogenic activity. However, in contrast to the inhibitory effect of a lower number of monocytes on tumour growth, no inhibition of tumour cell-induced angiogenesis was observed (Figure 5). Furthermore, tumour cell-preexposed $CD14^+$ cells injected with the HPC-4 cells at 1:1 and 0.1:1 ratios enhanced angiogenesis and the higher number of preexposed, but not the control, $CD14^+$ cells alone showed some proangiogenic activity (Figure 6).

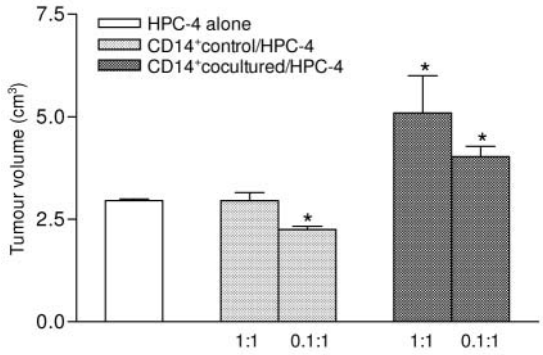

Figure 4. *The effect of CD14+ monocytes isolated from coculture with cancer cells on the HPC-4 tumour growth in SCID mice. Monocytes were cultured with HPC-4 cancer cells for 3 h, then CD14+ cells were isolated and coengrafted with HPC-4 cells at the ratio 1:1 and 1:0.1 into SCID mice. CD14+ cells isolated from monocytes kept in medium alone were used as control. Results are expressed as mean±SE of 5 different experiments. *Significantly different (p<0.05) in comparison with control.*

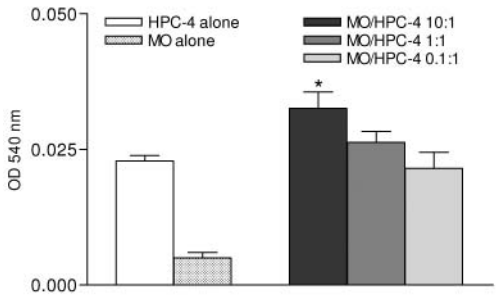

Figure 5. *The effect of different doses of monocytes (MO) on angiogenesis. MO and cancer cells at different ratios, MO at the highest dose and HPC-4 cells alone were mixed with Matrigel and injected s.c. into Balb/c mice. On day 6 matrigel was cut off and content of haemoglobin determined by absorbance at 540 nm. Results are expressed as mean±SE. *Significantly different (p<0.05) in comparison with control.*

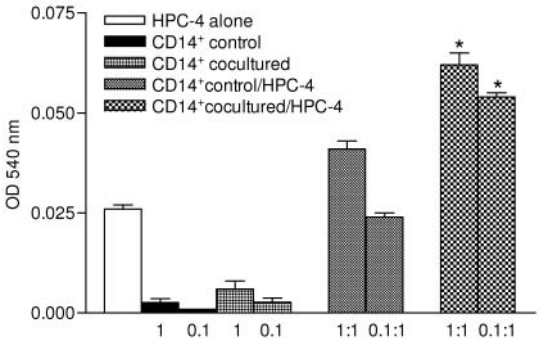

Figure 6. *The effect of different doses of CD14+ cells isolated from in vitro coculture with cancer cells on angiogenesis. CD14+ monocytes cultured in medium or cocultured with tumour cells were isolated by FACS sorting, mixed with Matrigel and injected s.c. together with cancer cells to Balb/c mice at 1:1 and 1:0.1 ratios. Haemoglobin content is expressed as absorbance at 540 nm±SD of 3 experiments. *Significantly different (p<0.05) in comparison with control CD14+ monocytes.*

Thus large numbers of untreated monocytes and lower numbers of preexposed monocytes enhanced the angiogenic activity induced by the tumour cells.

Discussion

This study provides the first direct numerical evidence of the effect of human monocytes on the growth of human tumour in SCID mice, demonstrating that monocytes may have opposite actions. The growth of HPC-4 tumours in SCID mice was enhanced by large numbers of monocytes and paradoxically inhibited when the monocyte/tumour cell ratio was low. This is compatible with clinical observations that abundant TIM infiltration, at least in some types of human cancer, is associated with poor prognosis (2, 3, 4) and *in vitro* data that at high monocyte/tumour K562 (erythroleukemia line) cell ratio the survival of the latter was enhanced, while at a lower ratio monocytes exhibited considerable cytotoxicity (19). However, it is unclear, also from this study, why this paradoxical effect occurs. It could be due to functional heterogeneity of monocyte subpopulations, deactivation or apoptosis of monocytes and/or production of a different range or quantity of cytokines by the monocytes. For example, $CD64^+$ monocytes exhibited enhanced tumour cytotoxicity in comparison to a $CD64^-$ subset (20). We have also recently shown that a $CD14^+/16^+$ monocyte subpopulation exhibited a significantly enhanced antitumour effect (21) and that preexposure of monocytes to tumour cells *in vitro* led to deactivation of their antitumour activity as evidenced by decreased cytotoxicity, ROI, TNF and IL-12 production and increased IL-10 release and apoptosis (8, 22). Such monocytes are analogous to TIM which represent a skewed M2 population, characterized by a $IL\text{-}12^- IL10^+$ phenotype, with protumour activity (6, 23). This may be supported by the present observations that monocytes preexposed *in vitro* to tumour cells before engraftment into SCID mice exerted a growth promoting effect on the tumour, even when used in small numbers. Furthermore, rather simplified analysis of cytokine levels in the coculture of monocytes at high ratio with tumour cells showed an enhanced production of IL-6 and IL-10 that are regarded as tumour promoting cytokines. Their levels were increased in the serum of some cancer patients (24) and were associated with shortened survival of patients with pancreatic (25) or hepatocellular (26) carcinoma. Since the HPC-4 and the monocytes at high

numbers cultured alone also produced IL-10 it may indirectly indicate, but not prove, that these cytokines are playing a role in the tumour growth enhancing effect of monocytes. Therefore, in the absence of the growth promoting effect of IL-6 and IL-10, the inhibition of tumour growth may occur at the low monocyte/tumour cell ratio.

Recently, the role of both these cytokines in the promotion of neoangiogenesis was defined (17, 18). The *in vivo* growth promoting activity of IL-6 for cervical cancer is associated with up-regulation of vascular endothelial growth factor (17). The role of IL-10 is more complicated as both a promoting (18) as well as an inhibitory (27) effect on angiogenesis has been reported. IL-10 may be produced in the tumour bed by infiltrating T lymphocytes, macrophages and tumour cells themselves. The present study showed that tumour cells exhibited significant angiogenic activity which was further enhanced when monocytes at high ratio were admixed to the tumour cells. However, monocytes deactivated *in vitro* showed some angiogenic activity and increased tumour cell-induced angiogenesis even when small numbers were used. This indicated that these cells express another property of M2 monocytes, that is the promotion of angiogenesis (7). Since M2 skewed monocytes are characterized by increased IL-10 production capacity (7, 8), it is likely that IL-10 may be responsible for the observed proangiogenic effect of deactivated monocytes.

In summary, the coengraftment of human monocytes and human cancer cells to SCID mice mimics the behaviour of TIM in clinical cancer demonstrating that monocytes in large numbers enhance tumour growth and angiogenesis. Deactivated, M2 polarized monocytes show an enhancing effect on tumour growth and tumour-induced angiogenesis. Despite some limitations this SCID model may be useful in studying complicated bidirectional interactions between macrophages and tumour cells and in defining cellular/molecular targets for the development of anticancer strategies.

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