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ORIGINAL ARTICLE

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Monocyte and monocyte-derived macrophage secretion of MCP-1 in co-culture with autologous malignant and benign control fragment spheroids

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Abstract This study was performed in order to determine how monocytes and macrophages in co-culture with autologous head and neck squamous cell carcinoma (HNSCC) tumor tissue regulate the secretion of monocyte chemotactic protein-1 (MCP-1). The levels of MCP-1 were measured when autologous monocytes or monocyte-derived macrophages (MDMs) were co-cultured in vitro with autologous fragment (F)-spheroids established from HNSCC tumors or benign mucosa serving as control. MCP-1 secretion from co-culture stimulated monocytes and MDMs was increased compared to spontaneous MCP-1 secretion. With prolonged co-culture, MDMs showed a steady-state MCP-1 secretion above background levels for up to 96 h, even with change of co-culture media every 24 h. Addition of an anti-MCP-1 antibody to the medium decreased co-culture-induced monocyte IL-6 secretion. Addition of lipopolysaccharide (LPS) (1 μg/ml) reduced MCP-1 secretion compared to spontaneous secretion in monocyte cultures. F-spheroids also secrete MCP-1, but at insignificant levels compared to the MCP-1 secretion from monocytes and MDMs. MCP-1 secretion from monocytes/MDMs is regulated differently when co-culture stimulation is compared to LPS-stimulation. Monocytes and MDMs expressed MCP-1 mRNA at a high level in all tested conditions: stimulated in co-culture, not stimulated or stimulated with LPS, indicating post-transcriptional regulation of MCP-1 secretion. The secretion of MCP-1 from tumor-derived F-spheroids, and the maintenance of co-culture MCP-1 secretion from MDMs in vitro, suggests that tumor-associated macrophages are a source of MCP-1 in HNSCC tumors.

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Introduction

Most carcinomas have the ability to metastasize hematogenously and form distant metastases. Distant metastases often preclude cure of the disease. The hematogenous spread of cancer cells may be impaired by cells of the immune system, because immune cells can act cytotoxically towards tumor cells. Examples of potential cytotoxic cells are natural killer (NK) cells [33], cytotoxic T-lymphocytes (CTL) [1] and, possibly, monocytes [3].

In both rodent and human models, monocytes and macrophages have been shown to bind in vitro and, if activated, kill tumor cells, via both direct contact and the secretion of cytotoxic active factors, such as TNF-α [5, 13]. In humans, the cytotoxic ability towards tumor cells has been shown to vary, depending upon the presence of autologous versus allogeneic monocytes and macrophages, and also to differ to some extent between individual patients with tumors of the same origin [11]. Because most studies on monocyte and macrophage interactions with tumor cells are performed at allogeneic conditions, and with highly selected tumor cell lines, inadequate information is available on how monocytes and macrophages act upon contact with spontaneously-developed autologous human carcinoma cells.

We have recently developed a model where autologous malignant cells and monocytes can be co-cultured in vitro [18]. Tumor tissue cut into fragments is cultured in flasks coated with agar, which inhibits adherence and subsequent fibroblast overgrowth. The fragments form free-floating rounded structures, named fragment (F)-spheroids, which then serve as vectors for autologous tumor cells/tumor cell products. Utilizing this technique, fragments of benign tissue harvested adjacent to the tumor can be maintained as controls in parallel cultures. We have shown that monocytes from consecutively included head and neck squamous cell carcinoma

(HNSCC) patients secrete IL-6, but not IL-1β or TNF-α when stimulated in vitro in co-cultures with autologous F-spheroids [18]. Both soluble substances, as well as direct contact between spheroids and monocytes, participate in the induction of this co-culture response [18].

Only a fraction of the monocytes are presumably in direct contact with the spheroids during co-culture. Yet, the co-culture response may be at the level of a generalized LPS-stimulated monocyte IL-6 response [18]. These findings suggest that the monocytes are stimulated by paracrine pathways in addition to actual co-culture stimuli.

Among factors having such an effect on monocytes is the monocyte chemotactic peptide 1 (MCP-1), a C-C chemokine originally shown to regulate macrophage recruitment into tumors [10, 34] and also shown to stimulate IL-6 secretion in monocytes [20]. MCP-1 is produced constitutively by monocytes isolated in vitro via adherence [26]. It is, therefore, of interest to investigate whether monocytes in co-culture with F-spheroids up-regulate MCP-1 secretion and whether addition of anti-MCP-1 to co-cultures inhibits the monocyte IL-6 response. Furthermore, MCP-1 secretion also attracts NK cells and T lymphocytes [2, 30]. Any up-regulated MCP-1 secretion could thus augment the cytotoxic response against tumor cells.

When blood-borne tumor cells establish micro-metastases in vivo, monocytes are exposed directly to the tumor cells before endothelial cells cover the metastases. Such monocytes are, presumably, precursors of tumor-associated macrophages (TAMs) [24] present especially in micro-metastases [25]. We have previously shown that the ability to produce IL-6 in response to co-culture stimuli in vitro is maintained for several days if monocytes mature to monocyte-derived macrophages (MDMs) in continuous co-culture with F-spheroids [19]. Therefore, it was pertinent to study whether macrophages secrete MCP-1 when co-cultured with F-spheroids, and if so, how.

Several cells in a solid tumor, e.g. endothelial cells, fibroblasts and tumor cells, potentially contribute to the recruitment of TAMs through MCP-1 secretion [12, 22, 15]. TAMs may also be a source of tumor-derived MCP-1. This assumption is supported by a positive correlation between MCP-1 activity level and percentage of TAMs shown in human tumors [4]. It was therefore of interest to determine to what extent virgin macrophages co-cultured with F-spheroids secrete MCP-1.

It is claimed that monocytes and macrophages act specifically towards malignant cells [13]. Therefore, co-cultures with F-spheroids established from benign control mucosa from the same patients were included in parallel experiments in order to determine whether the co-culture stimuli were dependent on malignancy-specific factors.

A link between the inflammatory and anti-tumor functions of macrophages has been proposed [8]. Therefore, the co-culture response was compared to that of lipopolysaccharide (LPS).

Material and methods

Patients

Patients with newly diagnosed squamous cell carcinomas of the oral cavity, oropharynx, hypopharynx, or larynx served as tissue donors when subjected to surgery. The study was approved by the Regional Committee for Medical Ethics at Haukeland University Hospital. Each patient gave written consent before participating in the study.

Fragment spheroid generation

An organ culture model was utilized to establish F-spheroids according to methods described previously [18]. In short, biopsies were obtained at surgery from tumor or benign control mucosa. The biopsies from the benign mucosa were chosen to resemble as closely as possible the tissue from which the tumor originated. Biopsies were cut into fragments with scalpels under dissection microscope. Cubes, with a size of 0.5–1.0 mm, were transferred to agar-coated tissue culture flasks (Nunc, Roskilde, Denmark) [14,28]. The fragments were cultured with Dulbecco's MEM (BioWhittaker, Walkersville, Md., USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS) (Sigma, St. Louis, Mo., USA), penicillin (100 IU/ml), streptomycin (100 µg/ml), amphotericin (2.5 µg/ml), L-glutamine (2 mM) and non-essential amino acid mixture (1%) (BioWhittaker) as culture medium. The cultures were maintained at 37 °C in 5% CO_2 and 95% air, with 100% relative humidity. Fragments that appeared as vital spheroidal structures after 10–14 days in culture were selected for experiments. These F-spheroids consist, as previously described, of epithelial cells, fibroblasts and macrophages [18]. Immunohistochemical staining has shown that the F-spheroids are devoid of lymphocytes (data not published).

Monocyte and MDM preparation

The peripheral blood mononuclear cells (PBMCs) were separated from blood by gradient centrifugation with Lymphoprep^R (Nycomed, Oslo, Norway) as density gradient medium [17]. The PBMC suspensions were at this stage devoid of granulocytes as analyzed by flow cytometry. The PBMC yield of 8.5 ml blood was allocated to a 24-well plate (Nunc) in RPMI-1640 supplemented as described for DMEM, but without non-essential amino acid mixture (RPMI complete) and with 20% autologous serum as serum source to a total volume of 0.5 ml/well. Monocytes were purified from PBMCs by 40 minutes of preliminary incubation, washed 3 times, and then cultured in RPMI complete/20% autologous serum; 0.5 ml/well. The monocytes were shown to be more than 95% pure, as assessed by non-specific esterase stain. Viability was more than 95%, as tested by trypan blue stain.

As macrophage source, monocytes were allowed to differentiate in vitro for time intervals up to 96 h in RPMI complete/10% or 20% autologous serum.

In vitro co-culture with F-spheroids and monocytes

Malignant (M) and benign (B) F-spheroids from 11 patients were washed and transferred to freshly-isolated monocyte cultures in 24 × 16 mm well plates (Nunc) with 0.5 ml RPMI complete with 20% autologous serum. Monocytes cultured in wells with RPMI/20% autologous serum with or without addition of lipopolysaccharide (LPS) (1 µg/ml) from *E. coli* (Sigma) served as controls. Two to six spheroids were allocated to each well. The monocytes or MDMs did not detach from the plastic surface nor adhere to or infiltrate the F-spheroids during the co-culture, as visualized by selective labeling of monocyte/MDM cultures with a fluorescent dye, PKH-26-PCL (Sigma) (not shown).

MCP-1 neutralizing antibody

To study the potential effect of MCP-1 on co-culture-stimulated monocyte IL-6 secretion, a monoclonal anti-human MCP-1 (neutralizing) antibody (R&D Systems) was added to the culture medium in one co-culture experiment. Experiments showed that addition of anti-MCP-1 did not inhibit the IL-6 LPS response of the monocytes. The monocyte IL-6 co-culture response in the presence of anti-MCP-1 (0.5 μg/ml or 10 μg/ml) was compared to the response at baseline co-culture conditions with the same F-spheroids and monocytes of the same patients.

Monocyte MCP-1 secretion after completed co-culture

In order to reveal whether MCP-1 in co-cultures is produced by monocytes or F-spheroids, the monocytes and F-spheroids were in one experiment cultured separately for another 24 h after 24 h of primary co-culture.

In vitro co-culture with F-spheroids and monocytes/MDMs

In order to study the time-dependent monocyte and MDM responsiveness, as measured by MCP-1 secretion, malignant (M) or benign (B) F-spheroids from 4 different patients were transferred to autologous monocyte or MDM cultures in 24-well culture plates (Nunc) with 0.5 ml RPMI complete/10% serum per well.

In one experiment, the monocyte/F-spheroid co-cultures were continued for 72 h and sampled at different time points. The media was either not changed, or changed every 24 h. In another experiment, the F-spheroids were subsequently transferred to new cultures of maturating monocyte/MDM cultures at 24, 48 and 72 h after initial monocyte isolation. The supernatants were subsequently sampled after 24 h of started co-culture. Monocyte/MDM cultures supplied with medium only, or medium supplied with LPS, served as control conditions in both experiments.

IL-6 and MCP-1 ELISA

The contents of IL-6 and MCP-1 were determined by ELISA, using MCP-1/IL-6 capture and detection antibody pairs, compared to r-hu IL-6/MCP-1 as standards (R&D Systems Europe, Abingdon, UK). All procedures were performed according to the specifications of the manufacturer. In short, 96-well microtiter plates (Costar Corning, N.Y., USA) were coated overnight at room temperature with monoclonal mouse anti-human IL-6 or MCP-1-capture antibodies, respectively. After blocking, diluted samples and respective recombinant human standards were added and incubated for 2 h at room temperature, followed by addition of biotinylated polyclonal goat anti-human IL-6 or MCP-1, respectively. The plates were incubated for 20 minutes at room temperature with streptavidin-conjugated horseradish peroxidase. Tetramethyl-benzidine (TMB) (Sigma) and H_2O_2 were used as substrate.

The background MCP-1/IL-6 levels from unstimulated cultures were subtracted from the levels of the parallel-stimulated cultures when the MCP-1/IL-6 levels are shown. MCP-1/IL-6 responses were in some experiments calculated as percent of appropriate LPS-stimulated monocyte response.

Contamination control

We did not detect the growth of any bacteria or fungi when samples of media from successfully completed co-cultures were tested on blood agar or Sabouraud glucose agar.

Endotoxin determination

Supernatants from cultures with F-spheroids incubated for 72 h were tested for endotoxin using the standard *Limulus* amoebocyte lysate assay (Associates of Cape Cod, Falmouth, Mass., USA). The samples were found to contain < 25 pg/ml LPS, which constituted the lower limit of the test.

mRNA purification

After co-culture was ended, the spheroids were removed and the supernatants harvested with the monocytes or MDMs still attached to the bottom of the wells. Monocytes and MDMs were then washed once with 0.5 ml cold PBS without Ca^{2+}/Mg^{2+} and each well lysed with 100 μl lysis/binding buffer (100 mM Tris-HCl, pH 8.0; 500 mM LiCl; 10 mM EDTA, pH 8.0; 1% lithium dodecyl sulfate; 5 mM dithiothreitol). Then, mRNA was isolated by magnetic polystyrene particles, Dynabeads Oligo $(dT)_{25}$ (Dynabeads® mRNA direct kit, Dynal, Oslo, Norway), as described [18]. The mRNA was stored at −80 °C until further analysis.

Semi-quantitative reverse transcription PCR

The expression of MCP-1 mRNA was determined semi-quantitatively by reverse transcription polymerase chain reaction (RT-PCR) using the GeneAmp EZ r*Tth* RNA PCR kit (Perkin Elmer, Roche Molecular Systems, N.J., USA.). To equalize the levels of mRNA in the samples, the RT-PCR reaction was first performed using samples of fivefold dilutions of mRNA in a final volume of 25 μl RT-PCR mixture, having a final concentration of 50 mM Bicine, 115 mM KC_2H_5O, 8% (w/v) glycerol (pH 8.2) as well as 300 μM of each dNTP, 2.5 mM $Mn(OA_c)_2$, 25 μM human β-actin primer pair and 5 U r*Tth* DNA polymerase (Perkin Elmer). Samples were then subjected to a thermo-cycler programmed at 60 °C for 30 min, followed by 40 cycles at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 45 s. The PCR products were analyzed by ethidium bromide-stained agarose-gel electrophoresis. Sample dilutions with equal expression levels of β-actin were used in a secondary RT-PCR, under the same reaction conditions as the primary RT-PCR, with β-actin (25 μM) and MCP-1 (0.6 pM) (R&D Systems) primer-pairs added in each sample.

The primers used were 5′-GGCACCACACCTTCTACAAT-3′ and 5′-AGGTAGTCAGTCAGGTCCCG-3‘ for β-actin and MCP-1 primer-pair manufactured by R&D Systems (RDP-24). The product size of cDNA for human β-actin and MCP-1 was 310 and 198 base pairs, respectively.

Statistics

The Statistical Package for the Social Sciences (Version 9.0; SPSS, Chicago, Ill., USA) was employed. The effect of presence versus absence of anti-MCP-1 on IL-6 monocyte co-culture response was compared by paired sample *t*-test. The same test was used when comparing monocyte MCP-1 response upon co-culture with malignant and benign F-spheroids, as well as LPS-stimulation and monocyte versus F-spheroid MCP-1 secretion. Differences were considered significant at $P < 0.05$.

Results

Effect of anti-MCP-1 antibody on co-culture-induced monocyte IL-6 secretion

An anti-MCP-1 antibody was added to the co-culture media at two different concentrations (0.5 μg/ml and 10 μg/ml) in order to test the effect of MCP-1 as a paracrine factor for monocyte IL-6 secretion in co-cultures. A decreased co-culture-induced monocyte IL-6 secretion was detected in four out of five separate

cultures with both 0.5 μg/ml and 10 μg/ml of anti-MCP-1 addition compared to the same cultures with malignant or benign F-spheroids but without additives to the culture media (standard) (Fig. 1). The reduced IL-6 co-culture response in the presence of anti-MCP-1 was significant when ln-transformed cytokine values were compared by the *t*-test ($P = 0.012$).

Comparison between co-culture and LPS-stimulated MCP-1 secretion by monocytes

Freshly isolated monocytes were co-cultured with malignant (M) and benign (B) F-spheroids from 11 different donors. Co-culture-stimulated monocytes secreted higher levels of MCP-1 than monocytes not stimulated in 19 out of 22 and 10 out of 11 individual cultures with M°F- and B°F-spheroids, respectively. Monocytes from respective donors showed a decreased MCP-1 secretion in 8 out of 11 LPS-stimulated cultures compared to spontaneous MCP-1 secretion (Fig. 2). A significant difference was found when mean MCP-1 co-culture responses with MF- and BF-spheroids from individual donors were compared by the paired samples *t*-test ($P = 0.003$). The same was true when the individual donor monocyte MCP-1 LPS-responses were compared to the respective mean monocyte MCP-1 co-culture responses with MF- and BF-spheroids ($P < 0.001$ and $P = 0.003$, respectively).

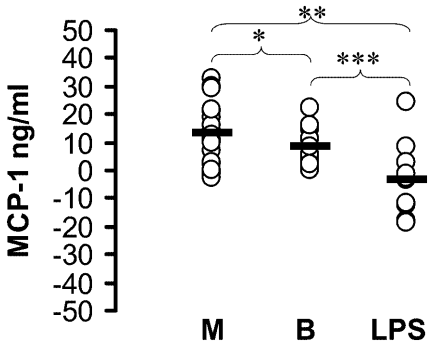

Fig. 2 Plots represent the levels of MCP-1 from 24 h in vitro co-cultures between autologous monocytes and malignant (*M*) or benign (*B*) F-spheroids, compared to MCP-1 levels in lipopolysaccharide (*LPS*)-stimulated monocytes in parallel cultures. The corresponding background MCP-1 secretion was subtracted. In these experiments, the cultures were supplied with RPMI-1640 with 10% autologous serum. In total, 23 malignant, 11 benign fragment spheroid co-cultures and LPS-stimulated monocyte cultures from 11 different donors were included. The MCP-1 levels were analyzed by ELISA. Statistics: co-cultures with BF-spheroids vs. MF-spheroids, $*P = 0.003$; LPS-induced vs. MF-spheroids co-culture, $**P < 0.001$; LPS-induced vs. BF-spheroids co-culture, $***P = 0.003$

Comparison between monocyte and F-spheroid MCP-1 secretion

Monocytes and F-spheroids from two different donors were separated after 24 h co-culture. MCP-1 levels in monocyte cultures separated from F-spheroids were reduced compared to levels in the primary co-cultures. The monocytes secreted MCP-1 at a level of approximately 64% of the co-culture response the following 24 h after initial co-culture, whereas the F-spheroid alone showed detectable MCP-1 levels only in five out of 12 cultures, with a mean of 3% of the primary co-cultures ($p < 0.000$, paired sample t-test) (Fig. 3).

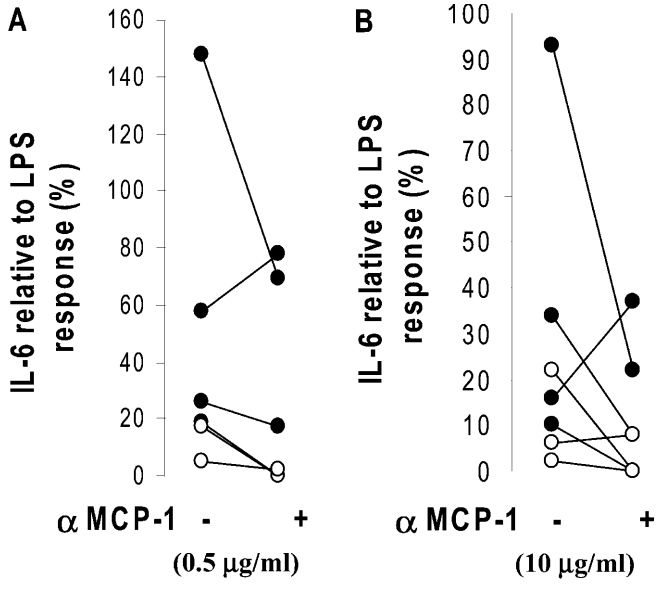

Fig. 1 Effect of adding anti (α)-MCP-1 antibody (0.5 μg/ml) (**A**) or (10 μg/ml) (**B**) on the levels of IL-6 in monocyte in vitro co-culture with either benign (○) or malignant (●) F-spheroids. The co-culture responses are calculated as fractions relative to LPS response in percent (%) after subtraction of respective spontaneous IL-6 secretions. Each line represents results from individual co-cultures with monocytes supplied with RPMI-1640/20% autologous serum in the absence (−) or presence (+) of α-MCP-1 at the given concentrations. Co-culture supernatants were analyzed for IL-6 by ELISA. Statistics: $*P = 0.012$ (ln-transformed) for co-culture response (−) compared to (+) α-MCP-1

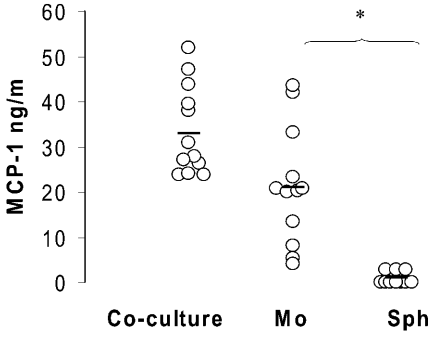

Fig. 3 Plots represent the levels of MCP-1 from primary 24 h in vitro co-cultures between autologous monocytes and BF- or MF-spheroids, followed by MCP-1 secretion from the corresponding monocytes (*Mo*) and F-spheroids (*Sph*) when separated into different cultures for the next 24 h. In this experiment, the corresponding background MCP-1 secretion was not subtracted. The cultures were supplied with RPMI-1640 with 10% autologous serum. Twelve cultures with monocytes and F-spheroids from two different donors were included. The MCP-1 levels were analyzed by ELISA. Statistics: monocyte compared to F-spheroid MCP-1 secretion, $*P = 0.000$

Comparison between in vitro co-culture and LPS-stimulated MCP-1 secretion by MDMs

Monocytes/MDM from 4 different donors were continuously stimulated in vitro with LPS (1 μg/ml) or by co-culture for 96 h. The supernatants were sampled every 24 h. MCP-1 levels in co-cultures were detected above background levels in supernatants after 20 h co-culture. The levels of MCP-1 increased linearly in all cultures (Fig. 4). LPS-stimulated cultures showed MCP-1 levels lower or equal to background MCP-1 levels, as indicated by negative values in three out of four cultures (Fig. 4). Changed medium every 24 h did not alter the MCP-1 secretion rate (Fig. 5).

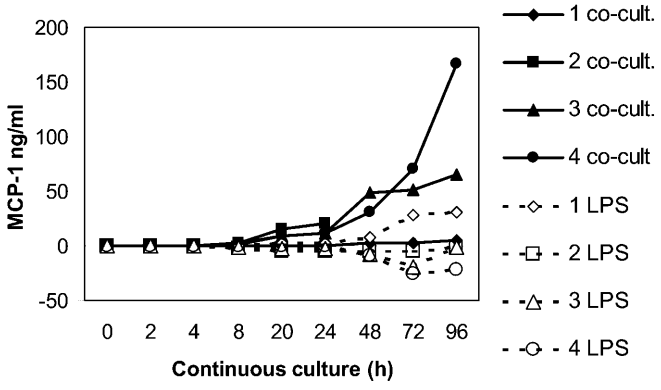

Fig. 4 Levels of MCP-1 in cultures of monocytes or monocyte-derived macrophages stimulated continuously in co-culture with MF-spheroids from 4 different patients (1–4) or by 1 μg/ml lipopolysaccharide (*LPS*) for the indicated number of hours (*h*). Each level of MCP-1 is adjusted for background secretion of MCP-1. In these experiments, cultures were supplied with RPMI-1640/10% autologous serum. MCP-1 levels were analyzed by ELISA

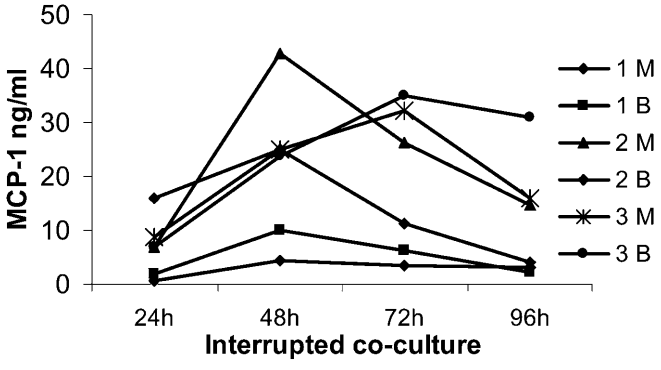

Fig. 5 Levels of MCP-1 in cultures of monocytes or monocyte-derived macrophages stimulated in co-culture with MF- or BF-spheroids for the indicated number of hours (*h*) and culture media (RPMI-1640/10% autologous serum) changed at 24, 48 and 72 h. Each sample was drawn after 24 h co-culture following media change. The figures show the results with cells from 3 different patients (*1–3*). Each level of MCP-1 was adjusted for the corresponding background MCP-1 secretion. MCP-1 levels were analyzed by ELISA

In vitro co-culture induced MCP-1 secretion in MDMs compared to monocytes

When F-spheroids were co-cultured for 24 h with MDMs formed by in vitro pre-incubation of monocytes from 24, 48 or 72 h, the MCP-1 co-culture response did not significantly change with increasing pre-incubation time (Fig. 6).

MCP-1 gene expression in monocytes and MDMs

mRNA was harvested from monocytes after 24 h of experimental conditions and from MDMs after 72 h pre-incubation, followed by 24 h of experimental con-

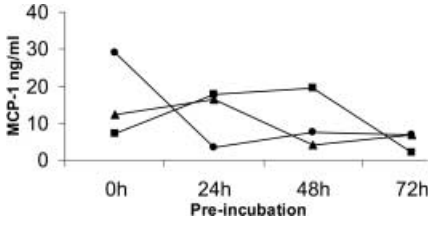

Fig. 6 Levels of MCP-1 in monocyte-derived macrophage cultures measured after 24 h stimulation in co-culture with RPMI-1640/10% autologous serum. Pre-incubation time before stimulation (0–72 h) is indicated on the x-axis. The figures show the results with cells from 3 different patients (■, ▲, ●) in co-culture with MF-spheroids. MCP-1 levels were adjusted for the background secretion of MCP-1. The supernatants of co-cultures were analyzed for MCP-1 by ELISA

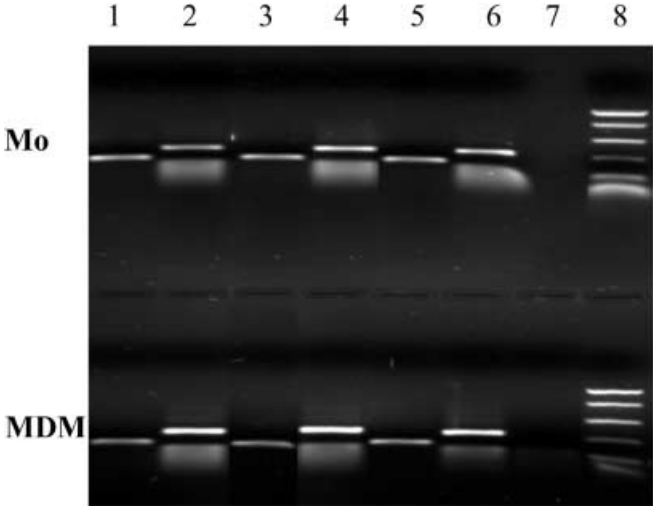

Fig. 7 MCP-1 and β-actin mRNA expression in monocytes (*Mo*) and monocyte-derived macrophages (*MDM*). After mRNA extraction, RT-PCR was carried out at fivefold dilutions with specific β-actin primers. Samples with equal levels of β-actin mRNA were selected. A secondary RT-PCR was performed using primers specific for MCP-1 and β-actin. Products were run on 1% agarose gels stained with ethidium bromide. The resulting bands were visualized by UV light. *Lanes 1,3,5* MCP-1 and *lanes 2,4,6* β-actin, *lanes 1–2* co-culture, *lanes 3–4* no stimulation, *lanes 5–6* LPS stimulation, *lane 7* absent mRNA and *lane 8* PCR markers. The figure shows one representative experiment out of three

ditions. The experimental conditions were: F-spheroid stimulation, no stimulation or LPS stimulation. The mRNA coding for MCP-1 was determined, together with β-actin mRNA expression, which served as control (Fig. 7). The MCP-1 mRNA expression was extensive and similar in monocytes and MDMs at all experimental conditions.

Discussion

Addition of anti-MCP-1 antibody to co-cultures reduced the monocyte IL-6 secretion compared to standard co-cultures. Furthermore, monocytes up-regulate MCP-1 secretion upon co-culture with autologous fragment (F)-spheroids from HNSCC patients in vitro. This indicates that MCP-1 secreted from co-culture-stimulated monocytes acts as a paracrine-stimulating factor that augments the co-culture-induced monocyte IL-6 secretion [7]. The increased MCP-1 secretion furthermore implies that monocytes may amplify the tumor cell cytotoxic potential of the immune system, primarily by attracting additional monocytes, and secondarily by attracting NK cells and T lymphocytes to metastatic tumor cells [2, 30].

The MCP-1 levels in co-culture supernatants could be derived from both the monocytes and the F-spheroids. The present experiments, however, show that secretion of MCP-1 from F-spheroids is present, but insignificant, compared to the co-culture monocyte/MDM-derived MCP-1. Therefore, the main source of MCP-1 in co-cultures is the monocytes or MDMs.

Monocytes/MDMs continued a steady-state MCP-1 secretion throughout a 96-h period with prolonged co-culture, indicating that monocytes infiltrating micro-metastases probably continue to secrete MCP-1 while differentiating towards TAMs. The continuous co-culture-induced secretion of MCP-1 from MDMs, as shown in this study, indicates that TAMs may augment the recruitment of both additional monocytes and other potential tumor cytotoxic cells towards tumor tissue, i.e. micro-metastases. The co-culture-induced MCP-1 secretion was, furthermore, similar to both monocytes and virgin MDMs employed in the co-culture. This points to an MCP-1 secretion from tissue macrophages upon contact with autologous tumor cells. Thus, TAMs may be one of the major sources of MCP-1 even in HNSCC primary tumors.

Change of medium every 24 h in prolonged co-culture did not influence the MCP-1 secretion from MDMs, indicating that the co-culture-induced MCP-1 secretion from MDMs is marginally dependent on autocrine stimulation once initiated.

The MCP-1 mRNA expression levels in both monocytes and MDMs were significant and similar when assessed after co-culture stimulation, in unstimulated monocytes and after 24 h of LPS stimulation. On the other hand, the differences in the level of MCP-1 cytokine secretion varied substantially. This suggests that the secretion of MCP-1 is post-transcriptionally regulated in both monocytes and MDMs with LPS as well as co-culture stimulation.

The mode of monocyte isolation as well as in vitro culture conditions, influence monocyte function [21]. The spontaneous secretion of MCP-1 by freshly isolated adherent monocytes is in line with observations made by others [26]. Platelets activated in cultures may also augment monocyte MCP-1 secretion via platelet-derived growth factor [23]. This must be acknowledged when interpreting the present results.

The density of TAMs in the tumor relate to prognosis of different types of carcinomas [6, 29]. Probably MCP-1, in part, recruits the cells. As shown in the present experiments, the MCP-1 secreted from HNSCC tumors may originate from the TAMs themselves. Thus, the tumor activation capacity of TAM MCP-1 production probably holds prognostic information. This would be in line with the significance of MCP-1 in macrophage recruitment, as well as in the host survival shown in human breast cancer [31].

Both F-spheroids from benign and malignant tissue stimulated monocytes and MDMs to secrete MCP-1. However, co-culture-induced monocyte MCP-1 secretion was increased with MF-spheroids compared to BF-spheroids. Thus, the interaction between a tumor and mononuclear phagocytes employs general pathways of activation, but the response level is modulated by a factor related to the malignant tumor.

MCP-1 is claimed to regulate monocyte chemotaxis in several types of inflammatory processes, i.e. wound healing [32], bacterial infections [9,26], rheumatoid arthritis [27], atherosclerosis [16], as well as cancer [4]. The recruitment of monocytes to a tumor or micro-metastases could thus be analogous to monocyte recruitment to an inflammatory site. The F-spheroid versus LPS-generated mononuclear phagocyte-generated MCP-1 secretion is, however, differentially regulated in other studies which is in accordance with findings in [26]. This suggests that the monocyte stimuli generated by tumor cells are different compared to gram-negative bacterial-generated inflammation. On the other hand, given the similarity between stimulation of monocytes and macrophages by FB- and FM- spheroids, we suggest that the tumor versus mononuclear phagocyte interactions resemble more of a tissue repair-like process than an infectious inflammation. Additional research needs to be done to answer these questions.

In conclusion, MCP-1 secretion from monocytes and MDMs is up-regulated when the cells are stimulated by F-spheroids as opposed to a down-regulation following LPS stimulation. MCP-1 augments co-culture-induced monocyte IL-6 secretion paracrinously. The co-culture-stimulated monocyte MCP-1 regulation is maintained during the in vitro maturation of monocytes to MDMs. Furthermore, virgin MDMs secrete MCP-1 in co-culture with F-spheroids, indicating that TAMs may be one source of MCP-1 in HNSCC tumors.

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