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Effects of calcitriol and its analogues on interaction of MCP-1 and monocyte-derived dendritic cells *in vitro*

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KEY WORDS dendritic cells; monocyte chemoattractant protein-1; calcitriol; calcipotriol; tacalcitol

ABSTRACT

AIM: To study the effect of calcitriol [1,25$(OH)_2D_3$] and its analogues on the interaction of monocyte chemoattractant protein-1 (MCP-1) and *in vitro* generated monocyte-derived dendritic cells (MoDC). **METHODS**: MoDC were obtained by differentiating monocytes in exposure to GM-CSF and IL-4 for 5 d. mRNA expression of MCP-1 and its receptors were analyzed by RT-PCR, and protein production of MCP-1 by ELISA and migratory ability of MoDC in response to MCP-1 by a micromultiwell chemotaxis chamber assay. **RESULTS**: MoDC can express MCP-1 mRNA, and secret a low level of MCP-1 protein and has the ability to migrate to MCP-1 in corresponding to its expression of MCP-1 receptors. Calcitriol and its analogues with the same affinity to vitamin D receptor up-regulated the gene expression of both MCP-1 and its receptors, enhanced MCP-1 protein production and promoted the migratory ability of MoDC to MCP-1. **CONCLUSION**: The interaction of DC and MCP-1 found in this study may suggest a possible autoregulatory role between DC and MCP-1 and the modulatory effect of calcitriol and its analogues on DC and MCP-1 might provide an understanding of their positive role in tumors.

INTRODUCTION

Calcitriol (1,25-dihydroxyvitamin D_3), a steroid hormone with a biological role in calcium homeostasis, is found to modulate growth and differentiation of a broad range of cells[1] and play an important role in the regulation of immune functions[2]. Large amount of investigations indicate that calcitriol exerted potent inhibitory effects on a variety of tumor cell lines of hemotopoietic or other origins by promoting differentiation, inhibiting proliferation, and inducing apoptosis[3-6]. In this context, the use of calcitriol and its synthetic analogues with less hypercalcemia as a putative therapy in tumors is postulated.

Recently, the biology of dendritic cells (DC) as a most potent antigen presenting cells in malignant disease is arousing great interest since the numbers of DC or Langerhans cell in or adjacent to tumors are found to correlate with tumor prognosis with increasing number of indicating better outcomes[7]. Monocyte chemoattractant protein-1 (MCP-1) is a chemotactic and activating factor for monocytes and is produced by multiple tumors and has antitumor effects[8]. In order to understand the relationship and interaction of the calcitriol, DC, and MCP-1 the following questions whether 1) DC can produce MCP-1; 2) DC migrate in response to MCP-1; 3) calcitriol modulates MCP-1 production by DC and the migratory activity of MCP-1 on DC are addressed here by us. An *in vitro* generated DC (monocyte-derived dendritic cells, MoDC) model was used in the present study and calcitriol and its analogues calcipotriol, tacalcitol, and 24,25$(OH)_2D_3$ were involved.

MATERIALS AND METHODS

Culture medium RPMI-1640 (BioConcept, Umkirch, Germany) supplemented with *L*-glutamine 2 mmol/L, 10 % fetal calf serum (BioConcept, Umkirch, Germany), penicillin 100 kU/L, and streptomycin 100 mg/L (Seromed, Berlin, Germany) was used as culture medium. Human recombinant IL-4 was kindly supplied by Essex Pharma (Munich, Germany), human recombinant granulocyte-macrophage colony-stimulating factor

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(GM-CSF) by Novartis Pharma (Nuenberg, Austria) and human recombinant MCP-1 purchased from BioConcept (Umkirch, Germany).

Chemicals Preservative-free calcitriol (Calbiochem-Novabiochem, Marburg, Germany), calcipotriol (kindly supplied by Leo Pharmaceuticals, Ballerup, Denmark), tacalcitol (supplied by Hermal, Reinbeck, Germany) and $24,25(OH)_2D_3$ (kindly supplied by Schering, Berlin, Germany) were dissolved in 96 % analytical grade ethanol to a concentration of 1 mmol/L and further diluted in culture medium.

MoDC culture Highly enriched blood monocytes (>90 % $CD14^+$ by FACS determination) were obtained from buffy coats (through the courtesy of Transfusion Center of Kiel University, Germany) by density gradient centrifugation over Ficoll/Paque (Biochrom, Berlin, Germany) and purified by counterflow centrifugation elutriation (J2-21M/E Beckman, Munich, Germany). Monocytes were cultured for up to 5 d at a cell density of 2×10^3 cells/L (3 mL in each well) in six-well tissue culture plates (Becton Dickinson, Heidelberg, Germany) in culture medium at 37 ℃ in a 5 % humidified CO_2 atmosphere. The resultant population of cells has been extensively characterized morphologically, phenotypically, and functionally (data not shown).

Calcitriol, calcipotriol, tacalcitol, and 24, 25-$(OH)_2D_3$ were added to the cell culture from the beginning with a concentration of 0.01 μmol/L. An ethanol-solvent control (0.01 %) was included at the same time.

RT-PCR analysis and ELISA detection Total RNA was extracted from cells harvested after 5-d culture using TRIzol reagents (Gibco-BRL, Karlsruhe, Germany). Each sample of RNA (3 μg) was reverse transcribed into cDNA with oligo(dT) and the cDNA was amplified by PCR techniques with Taq Polymerase (Gibco-BRL, Karlsruhe, Germany) and PCR buffer. MCP-1 (upper: GACCCTACAAAACTCCCAGAACAGCTGAATT; lower: TAACCTACAAAGACCCAATCAGCTGAAACC) and MCP-1 receptor A and B primers were kind gifts from Dr J BARTELS (Department of Dermatology, University of Kiel, Germany). Amplification of the house-keeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a generous gift from Dr C SCHLUETER (Department of Dermatology, University of Kiel, Germany) was always involved to serve as control of reaction efficacy. For determination of MCP-1 production, supernatants were collected from MoDC and stored at −70 ℃ until measurement. ELISA method was used with a commercially available kit from R&D Systems (Wiesbaden, Germany) according to the manufacture's protocols.

Migration assay Cell migration was assessed by a 48-well microchamber technique[9]. Twenty eight microliters of chemoattractant solution or control medium (RPMI-1640 with 10 % FCS) used for assaying random migration were added to the lower wells of a chemotaxis chamber (Costar, Bodenheim, Germany). Fifty microliters of 5-d cultured MoDC suspension (cell concentration = 100 L^{-1}) were seeded in the upper chamber. The two compartments were separated by a 5-μm pore size polyvinylpyrrolidone-free (PVPF) membrane (Costar, Bodenheim, Germany). The chamber was incubated at 37 ℃ in humidified atmosphere in the presence of 5 % CO_2 for 2 h. At the end of the incubation, filters were removed, fixed with ethanol, and stained with hemotoxylin solution (Sigma, Steinheim, Germany). Migrated cells on the lower side of the polycarbonate membrane were quantified after staining by measuring specific light absorbance using a computer controlled densitometer (CD60 4.1, Desaga, Heidelberg, Germany). Results are expressed as a chemotactic index (CI) by calculating the mean degree of absorbance of the area covered by migrated cells following stimulation with chemoattractant divided by the mean degree of absorbance of the area covered by unstimulated randomly migrated cells[10]. Each experiment was performed in duplicate.

Statistical analysis Data were expressed as $\bar{x}\pm s$ obtained from at least 3 independent experiments, and assessed by t test.

RESULTS

Expression of MCP-1 mRNA and production of MCP-1 protein As shown in Fig 1 and Fig 2, 5-d cultured MoDC expressed MCP-1 mRNA and secreted a low level of MCP-1 protein. Calcitriol and its analogues calcipotriol and tacalcitol which possessed the same affinity to vitamin D receptor (VDR) up-regulated both mRNA expression and protein production of MCP-1 with the same extent, however, the solvent ethanol and another calcitriol analogue $24,25(OH)_2D_3$ which has very low affinity to VDR than calcitriol showed no effect.

Expression of MCP-1 receptor mRNA Expression of MCP-1 receptor A and B mRNA were detectable by RT-PCR in MoDC. With the treatment of calcitriol and tacalcitol at a concentration of 0.01

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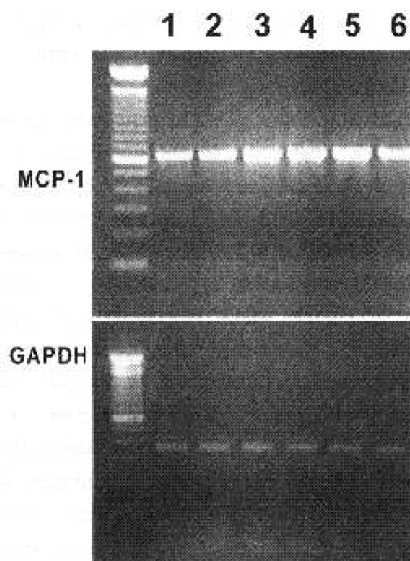

Fig 1. **Expression of MCP-1 generated by MoDC *in vitro*. Medium control (lane 1), 0.01 % ethanol (lane 2), calcitriol (lane 3), calcipotriol (lane 4), tacalcitol (lane 5), and 24,25(OH)$_2$D$_3$ (lane 6) at a concentration of 0.01 μmol/L. GAPDH, internal standard. One experiment representative of three was demonstrated.**

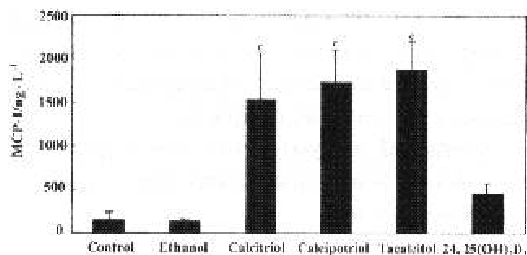

Fig 2. **Production of MCP-1 protein by MoDC in the presence or absence of calcitriol, calcipotriol, tacalcitol, and 24,25(OH)$_2$D$_3$ at a concentration of 0.01 μmol/L. n=5. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control group.**

μmol/L, the signal of expression became stronger (Fig 3). A micromultiwell chemotaxis chamber assay was used to investigate the ability of MoDC to migrate in response to MCP-1. Migration of MoDC to MCP-1 was observed at concentrations from 0.001 nmol/L to 10 nmol/L and the result showed that MCP-1 exhibited weak chemotactic activity for DC (Fig 4). Calcitriol and tacalcitol at a concentration of 0.1 nmol/L and 1 nmol/L enhanced the migratory ability of MoDC to MCP-1 in corresponding to their up-regulatory effect on the MCP-1 receptor expression (Fig 4). Ethanol and 24,25(OH)$_2$D$_3$ showed no discernable effect.

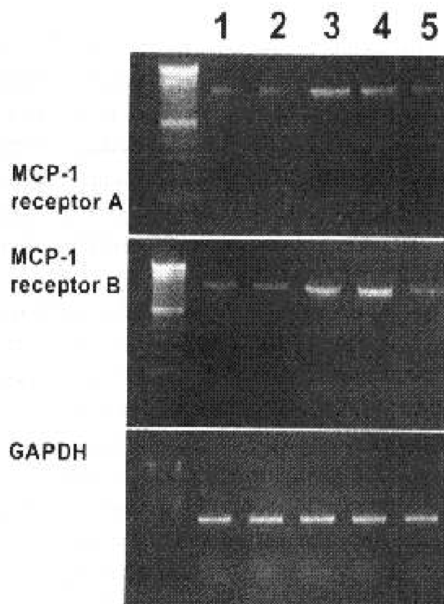

Fig 3. **Expression of MCP-1 receptor A and B generated by MoDC *in vitro*. Medium control (lane 1), 0.01 % ethanol (lane 2), calcitriol (lane 3), tacalcitol (lane 4), and 24,25(OH)$_2$D$_3$ (lane 5) at a concentration of 0.01 μmol/L. GAPDH signal was shown as interanl standard. One experiment representative of three was demonstrated.**

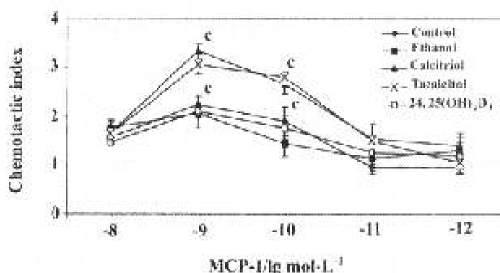

Fig 4. **Chemotactic activity of MCP-1 for MoDC treated with or without ethanol (0.01 %), calcitriol, tacalcitol, and 24,25(OH)$_2$D$_3$ (0.01 μmol/L). Results were obtained from 3 experiments and expressed as chemotactic index (CI). n = 3 (duplicate). $\bar{x} \pm s$. $^cP < 0.05$ *vs* control group.**

DISCUSSION

Since the early 1980s, in addition to epidemiological study showing a relationship between vitamin D and can-

cer, an increasing amount of cell biological data have demonstrated that at high concentration (1 – 100 nmol/L) calcitriol inhibited the growth of tumor cells *in vitro* such as malignant melanoma cells[4] and myeloid leukemia cells[11]. These *in vitro* findings were followed by the observation that calcitriol has beneficial effects in several *in vivo* models of tumors, for example, breast cancer[12], colon cancer[13], lung cancer[14], and melanoma[13]. Thus, the development of treatment strategies based on vitamin D_3 in a wide range of cancers is promising. But the mechanism of action is unclear up till now, although it is believed to be mediated by VDR distributed in a variety of tumors[15].

In the present study, an *in vitro* generated DC model and MCP-1 are used as the target to investigate the effect of calcitriol for the reason that increased numbers of DC are demonstrated to be associated with better prognosis in colorectal adenocarcinoma[16], lung adenocarcinoma[17], cutaneous T cell lymphomas[18], and so on, and MCP-1 is verified to be expressed and produced by multiple tumors and is believed to have antimutor effects as well[8]. Therefore, both DC and MCP-1 are likely to have beneficial effect against tumor cells. It is expected that there may be some interactions between DC, MCP-1, and calcitriol. With RT-PCR and ELISA method we found that *in vitro* generated MoDC expressed MCP-1 mRNA (Fig 1) and produced a low level of MCP-1 (Fig 2) and at the same time they expressed MCP-1 receptor mRNA (Fig 3) which is prerequisite for cells to migrate in response to its ligand. The migration assay verified that MCP-1 had chemotactic acitivity on MoDC (Fig 4). The ability of DC to express and produce MCP-1 along with the expression of MCP-1 receptor and the consequent migration in response to MCP-1 suggests a possible autoregulatory mechanism in the interaction of DC and MCP-1, thus if either DC or MCP-1 exists in or adjacent to tumor tissues they will contribute to the better prognosis. This understanding is helpful to give a theoretical basis in developing the use of dendritic cells against cancer which is in recent years a burgeoning field of research. Calcitriol, though is a hopeful agent for treating cancer there is a risk of hypercalcemia. Analogues are synthesized to exert the same therapeutic effect as calcitriol but to have minimized calcitropic activities. Calcipotriol, tacalcitol, and 24,25$(OH)_2D_3$ are analogues of calcitriol with the former two having same potent affinity to VDR as calcitriol while 24,25$(OH)_2D_3$ has very low affinity to VDR. In our experiment, calcitriol and its analogues calcipotriol, tacalcitol, and 24,25$(OH)_2D_3$ were included and were observed with a concentration of 0.01 μmol/L. The result showed that calcitriol and its analogues except 24,25$(OH)_2D_3$ up-regulated the expression of MCP-1 mRNA, secretion of MCP-1 protein, and expression of MCP-1 receptor mRNA by MoDC. The result of our chemotaxis assay that MCP-1 had weak chemotactic ability for MoDC (Fig 4) indicates MoDC themselves do not have abundant receptors of MCP-1. Calcitriol 0.01 μmol/L and tacalcitol but not 24,25$(OH)_2D_3$ enhanced the mRNA expression of MCP-1 receptor by MoDC (Fig 3). Correspondingly, the migratory ability of calcitriol and tacalcitol but not 24,25$(OH)_2D_3$ treated MoDC to MCP-1 1 nmol/L and 0.1 nmol/L was enhanced. But this modulatory effect was not seen when MCP-1 was used at a concentration of 0.01 μmol/L (Fig 4). This may suggest that MCP-1 0.01 μmol/L is too high a concentration for the receptors bound on MoDC resulting in chemokinesis (accelarated but nondirectional migration) rather than chemotaxis. These data indicate that calcitriol and its analogues with the same affinity to VDR may be useful in the treatment of cancer by enhancing the putative autoregulatory role between DC and MCP-1 which is important in tumor prognosis. The undetectable effect of 24,25$(OH)_2D_3$ in this issue provides an indirect evidence that VDR is the crucial point of calcitriol to exert its activities.

Taken together, our *in vitro* results show that calcitriol and its analogues can enhance a possibly existed auto-regulatory role between DC and MCP-1. These results might point to the understanding how DC, MCP-1, and calcitriol play their positive role in tumors.

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骨化三醇及其类似物对MCP-1与单核细胞衍生的树突状细胞间相互作用的影响

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关键词　树突状细胞；单核细胞趋化蛋白-1；骨化三醇；卡泊三醇；他卡西醇

目的：研究骨化三醇及其类似物对单核细胞趋化蛋白-1(MCP-1)和单核细胞衍生的树突状细胞(MoDC)两者之间相互作用的影响．**方法**：用GM-CSF和IL-4使单核细胞经体外培养5天后分化为MoDC．用RT-PCR分析MCP-1及其受体的mRNA表达，ELISA测定蛋白质水平．用微孔化学趋化板检测MoDC对MCP-1的游走功能．**结果**：MoDC能表达MCP-1 mRNA，分泌低水平MCP-1蛋白，并表达MCP-1受体从而具有向MCP-1游走的功能．骨化三醇及其类似物卡泊三醇和他卡西醇对维生素D受体具有相同亲和力，它们均能上调MoDC的MCP-1和MCP-1受体的表达，增加MCP-1蛋白质水平并促进MoDC对MCP-1的游走功能．**结论**：树突状细胞(DC)与MCP-1间可能存在自调节作用．骨化三醇及其类似物对DC和MCP-1的调节可使其在肿瘤治疗中起积极作用．

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