Expression of the cell-adhesion molecule VCAM-1 by stromal cells is necessary for osteoclastogenesis

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Abstract Osteoblastic cells have been shown to be involved in osteoclast formation through cell to cell contacts. This study was designed to examine the possible function of vascular cell adhesion molecule 1 (VCAM-1) during osteoclastogenesis. As a source for stromal cells we used the recently established mouse bone marrow stromal cell line mBMS-B1 which has the ability to support osteoclastogenesis when used in co-culture with a crude spleen cell suspension. mBMS-B1 cells express a single ~3.9 kb VCAM-1 mRNA species. Expression was low under basal culture conditions and a 5–10-fold increase was observed in the presence of $1,25(OH)_2D_3$. Cell surface expression of VCAM-1 examined by FACS analysis was increased about 2-fold after $1,25(OH)_2D_3$ treatment. Immunoprecipitation of cell surface expressed VCAM-1 or total VCAM-1 protein using the anti-VCAM-1 monoclonal antibody MK2.7 resulted in a single ~110 kDa protein on SDS-PAGE. Induction by $1,25(OH)_2D_3$ was about 2–5-fold on day 3. The stromal cell–osteoclast precursor cell interaction was investigated in a co-culture of the mBMS-B1 and mouse spleen cells in the presence of $1,25(OH)_2D_3$. The monoclonal antibody MK2.7 which is known to block hemopoietic-stromal cell recognition inhibited the formation of osteoclasts when added to the co-culture at day 2 but not day 4. These data suggest that VCAM-1 is involved in the interaction between stromal cells and osteoclastic precursor cells during osteoclastogenesis presumably most important during early stages of the formation of osteoclasts.

Key words: Osteoclast development; Stromal cell; Cell adhesion; VCAM-1

1. Introduction

During the development of osteoclasts, the osteoclast precursor cells are considered to interact closely with osteoblasts and/or stromal cells [1]. In a co-culture system between mouse spleen cells and a mouse osteoblastic cell line, cell to cell contacts were shown to be involved in osteoclastogenesis and the osteoblastic cell line could be replaced by stromal cell lines derived from bone marrow [2,3]. Cell adhesion molecules on stromal cells could be important for the release of cytokines as well as for mediating undifferentiated bone marrow cell binding. Recently, it has been shown that LFA-1 and ICAM-1 interactions are involved in tumours in the formation of multinucleated giant cells (MGC) which like osteoclasts are formed through the differentiation and fusion of progenitors [4]. Further studies indicated that LFA-1 and ICAM-1 may play a role in osteoclast development via interaction between stromal cell and osteoclast progenitors as well as among osteoclast progenitors [5]. ICAM-1 and VCAM-1 are also expressed on osteoblasts and crosslinking of these adhesion molecules on the osteoblast's cell surface induced IL-6 secretion from the osteoblasts [6]. In the present study we wanted to investigate the impact of VCAM-1 expression on stromal cells during osteoclastogenesis using a co-culture system between the recently established murine stromal cell line mBMS-B1 [7] and murine spleen cells. VCAM-1 was first identified as an adhesion molecule inducible on endothelial cells by inflammatory cytokines such as IL-1, tumour necrosis factor and lipopolysaccharide and it stays stably expressed at the cell surface for more than 48 h after induction. The molecule binds to a variety of leukocytes, including B-cells, T-cells, basophils, eosinophils, monocytes and lymphocyte precursors. VCAM-1 belongs to the Ig superfamily and several splice variants have been described whereas the major form in humans contains seven Ig domains of which domains 1–3 are homologous to domains 4–6 [8,9]. The binding partner of VCAM-1 is the β_1 integrin VLA-4 which is found on all leukocytes except neutrophils [10,11]. As $1,25(OH)_2D_3$ is necessary for the development of osteoclasts in our co-culture system we also investigated the influence of $1,25(OH)_2D_3$ on VCAM-1 expression in the mBMS-B1 stromal cells.

2. Materials and methods

2.1. Cell culture

mBMS-B1 cells were cultured in DMEM/Ham's F12 (1:1) supplemented with 100 U/ml of benzylpenicillin, 100 μg/ml streptomycin and 10% (v/v) FCS at 33°C [7].

2.2. Immunoprecipitation

mBMS-B1 cells (3×10^6) were labelled for 4 h with 200 μCi of [^{35}S]methionine in 1 ml of methionine-free MEM. The labelling medium was added to the cells 1 h before addition of the radioactive amino acid. Immunoprecipitations were done as described [7].

For cell surface immunoprecipitations, 3×10^6 radioactively labelled mBMS-B1 was washed in ice-cold PBS and incubated in the culture dish for 45 min at 4°C with the mAb MK2.7 (50 μg/ml). Antibodies were washed away by rinsing the culture dish three times with PBS. Cells were scraped off the plate with a rubber policeman, and the pelleted cells were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM phenylmethylsulfonyl fluoride; 0.5% Triton X-100). The lysis buffer already contained the extracted cell proteins of a 10-fold excess of unlabelled mBMS-B1 cells. Further immunoprecipitation was carried out as usual.

2.3. Flow cytometry

Cells were grown to confluence and incubated with 10^{-8} M $1,25(OH)_2D_3$ for the periods indicated in the figure. Cells were detached with 10 mM EDTA/PBS and reacted with the anti-VCAM-1 antibody MK2.7 and MK1.9 [12]. After 1 h incubation at 4°C, cells were washed and incubated with the anti-rat fluorescinisothiocyanate (FITC)-conjugated second antibody. Background staining was assessed in the presence of the second antibody only. Five thousand viable cells were analyzed with a FACscan (Becton Dickinson).

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2.4. RNA blot-hybridization (Northern) analysis

Total RNA was prepared and analyzed by hybridization to the ^{32}P-labelled murine VCAM-1 DNA probe using standard procedures [13].

2.5. Bone resorption–Pit assay

Dentine slices (4×4 mm, thickness 130–180 mm) were prepared with a low-speed diamond saw (Buehler, Illionis, USA), sterilized with ethanol and kept in culture medium for at least 2 h prior to use. Stromal cells were seeded in 48-well plates on the dentine slices and grown to confluence. After 2 days at 39°C, 5×10^5 a crude spleen cell suspension, prepared from 8–14-day-old mice, was added per well. Culture was continued in the presence of $1,25(OH)_2D_3$ (10^{-8} M) and dexamethasone (10^{-7} M) for further 12 days. At the end of the culture period, the slices were washed twice with distilled water and the cells were removed from the slices by rubbing with a silicon rubber stop. The slices were etched in 5% (w/v) aluminum sulfate for 10 min, rinsed with distilled water, stained with Coomassie brilliant blue (0.5% CBB in 45% (v/v) methanol, 9% (v/v) acetic acid) for 2 min, rinsed in water and embedded. Resorbed area was quantified using a Leitz Quantimet image analysis system and expressed as mm^2 resorbed area per cm^2.

3. Results and discussion

The expression of the cell adhesion molecule VCAM-1 by the stromal cell line mBMS-B1 was first examined by FACS analysis. mBMS-B1 cells clearly show a basal expression of VCAM-1 as they react with the anti-VCAM-1 mAb MK2.7 (Fig. 1). In the same experiment the influence of $1,25(OH)_2D_3$ on the VCAM-1 expression was investigated. Cells were grown to confluence at 33°C and further cultured in the presence or absence of $1,25(OH)_2D_3$ at 37°C. In the presence of 10^{-8} M $1,25(OH)_2D_3$ VCAM-1 is up-regulated about 1.5–2-fold and the induction sustains during the entire observation period of 8 days.

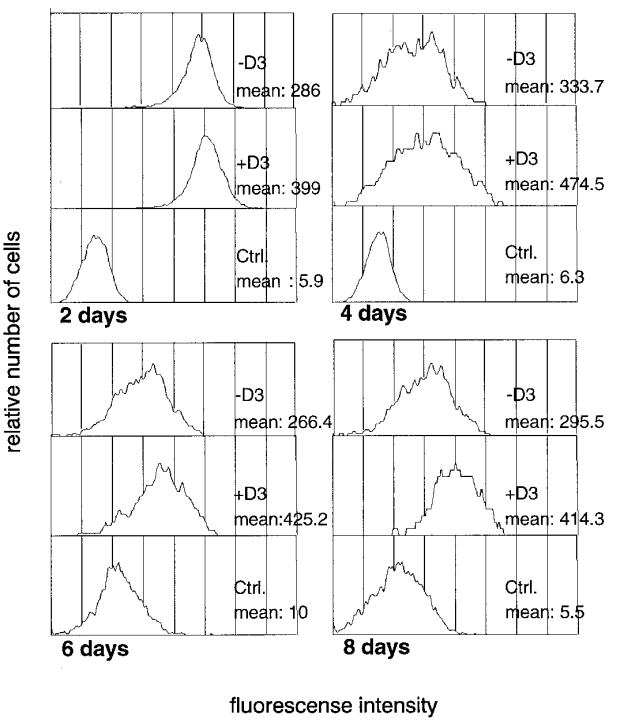

Fig. 1. Cell surface expression of the cell adhesion molecule VCAM-1. Clone mBMS-B1 was grown to confluence and treated with or without 10^{-8} M $1,25(OH)_2D_3$ (−D3 or +D3) at 37°C for the time period indicated. Thereafter cells were processed for FACS analysis using the anti-VCAM-1 mAb MK2.7. The mean value of the fluorescence are indicated. Ctrl.: cells incubated with second antibody only. A representative experiment of two different determinations is shown.

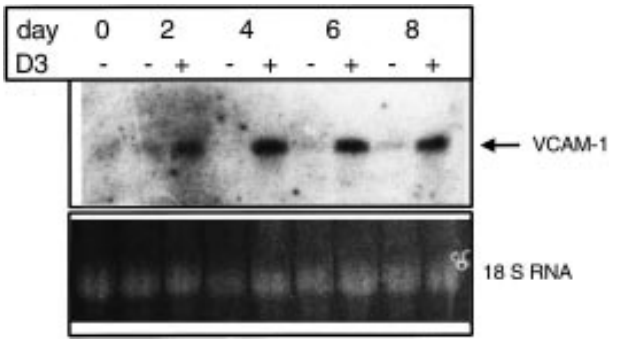

Fig. 2. Induction of VCAM-1 mRNA by $1,25(OH)_2D_3$. mBMS-B1 cells were grown to confluence (day 0) at 33°C or further cultured at 37°C for 2, 4, 6 or 8 days in the presence or absence of $1,25(OH)_2D_3$ (D3). Thereafter total RNA was extracted and 15 μg loaded per lane. The cDNA probe used for hybridization was murine VCAM-1. A representative experiment of two different determinations is shown.

To clarify if the up-regulation of VCAM-1 by $1,25(OH)_2D_3$ takes place at the level of transcription or translation we carried out Northern blot analysis (Fig. 2). mBMS-B1 cells were grown to confluence at 33°C and further cultured in the presence or absence of $1,25(OH)_2D_3$ at 37°C for the times indicated in the figure. Northern blot analysis of total mRNA using a murine VCAM-1 probe showed a single ~3.9 kb VCAM-1-specific mRNA species. The basal expression of the VCAM-1 mRNA seems to be very low and is not induced after postconfluent growth of the mBMS-B1 cells. Treatment of the mBMS-B1 cells with $1,25(OH)_2D_3$ dramatically enhances the expression of VCAM-1 mRNA indicating that the induction mainly takes place on the transcriptional level. Quantitative analysis using a PhosphorImager shows that the increase in steady-state mRNA is about 5–10-fold (data not shown). The increase is sustained during the entire observation period of 8 days. These results demonstrate the absence of VCAM-1 splice variants in the mBMS-B1 cells which had been described in other cell lines [8,9,14,15]. In the mouse a glycolipid-anchored, truncated splice variant of VCAM-1 comprising only the first three Ig domains has been reported [16,17].

Next we investigated the VCAM-1 expression on protein level (Fig. 3). Immunoprecipitation of cell surface-expressed VCAM-1 or total VCAM-1 protein using the anti-VCAM-1 monoclonal antibody (mAb) MK2.7 resulted in a single ~110 kDa protein on SDS-PAGE. Quantivative analysis of the bands using a PosphorImager indicated that the induction of VCAM-1 by $1,25(OH)_2D_3$ was about 3–5-fold on protein level (data not shown). The elevated expression of VCAM-1 after $1,25(OH)_2D_3$ treatment was visible in both cases, namely after precipitation of VCAM-1 from total cell lysate (compare lanes 1 and 2) and in the cell surface expressed VCAM-1 fraction (compare lanes 3 and 4). In line with the results gained from the Northern blot analysis no truncated splice variant of VCAM-1 could be detected. Furthermore, no glycosylated variants of VCAM-1 which had been described for the glycolipid anchored VCAM-1 in mouse endothelioma cells [14] seem to be present in the mBMS-B1 cells.

Finally we were interested in the functional impact of the VCAM-1 expression and VCAM-1 inducibility on osteoclas-

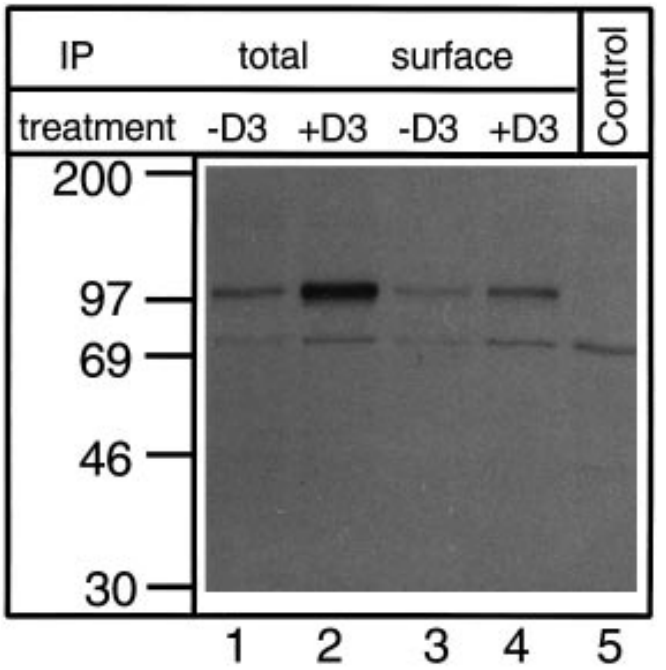

Fig. 3. Induction of VCAM-1 by $1,25(OH)_2D_3$ on protein level. mBMS-B1 cells were grown to confluence at 33°C and incubated for 3 days at 37°C in the presence or absence of 10^{-8} M $1,25(OH)_2D_3$ (D3). Thereafter cells were labelled for 4 h with [^{35}S]methionine and either detergent extract (lanes 1 and 2) or intact cells (lanes 3 and 4) were incubated with mAb MK2.7. For cell surface immunoprecipitations intact cells were washed after antibody incubation and lysed in detergent buffer containing a 10-fold excess of unlabelled mBMS-B1 cellular protein. Antigen antibody complexes were pelleted with protein G–Sepharose and separated on a 10% polyacrylamide gel. A representative experiment of two different determinations is shown.

togenesis. We have recently demonstrated that the mBMS-B1 cells have the ability to support the induction of osteoclasts from a crude spleen cell population using co-culture experiments [7]. This osteoclast inductive capacity was only observed at the non-permissive temperature for cellular growth at 39°C and in the presence of 10^{-8} M $1,25(OH)_2D_3$. To examine the influence of VCAM-1 on osteoclastogenesis we carried out co-culture experiments in the presence or absence of the VCAM-1 specific mAb MK2.7 which blocks the interaction between VCAM-1 and its ligand (Fig. 4). At the end of the culture, bone resorption pits were identified on the dentine slices as a measurement for the number of osteoclasts formed. Formation of osteoclasts from the precursor cells is dose-dependently inhibited by mAb 2.7. The mAb MK1.9 which binds to VCAM-1 but does not block the interaction with the ligand does not significantly influence osteoclastogenesis. Furthermore, when the blocking mAb MK2.7 was added at day 4 of co-culture, influence on the osteoclast formation could no longer be observed.

First, these data clearly indicate that a cell to cell contact between the stromal cell line mBMS-B1 and the osteoclast precursor cells is necessary for osteoclastogenesis. The cell adhesion molecule VCAM-1 expressed on the stromal cell and the corresponding ligand on the osteoclast precursor cell are crucial for osteoclast formation. The induction of VCAM-1 upon the addition of $1,25(OH)_2D_3$ might improve the interactions between both cell types in the co-culture. It is well established that in several co-culture systems the addition of $1,25(OH)_2D_3$ is absolutely required for the formation of osteoclasts [2,7]. Therefore, further factors induced by $1,25(OH)_2D_3$ might be involved in osteoclastogenesis. The interaction of VCAM-1 with its ligand on the osteoclast precursor cells might, in addition to the function as a glue to the opposing partner, transduce activation signals in the stromal cells which facilitate the production of factors stimulating osteoclastogenesis, a concept which has recently been described [6].

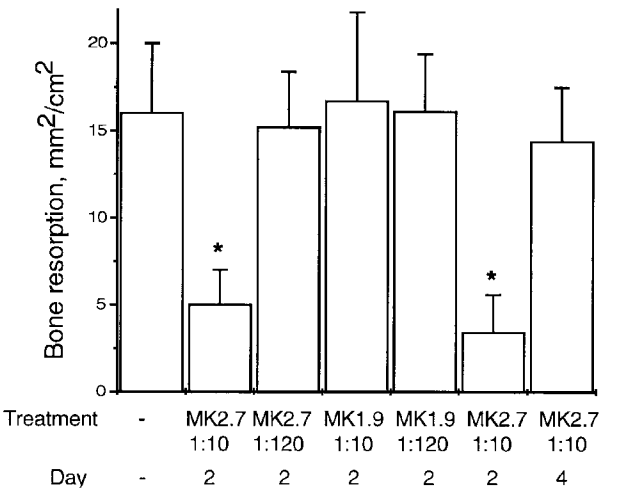

Fig. 4. Blocking anti VCAM-1 antibody inhibit osteoclast formation in the co-culture system. Stromal cells were seeded in 48-well plates on the dentine slices and grown to confluence at 33°C. After 2 days at 39°C, 5×10^5 mouse spleen cells were added per well. Culture was continued at 39°C in the presence or absence of the mAb for further 10 days. Antibody solutions were purified hybridoma supernatant 1:10 or 1:120 diluted. Data are shown as mean ± SD of at least four different determinations. Experiments were performed three times with similar results. $^*P<0.01$ compared with corresponding non-mAb-treated group.

Second, the interaction between VCAM-1 and its ligand on the osteoclast precursor is necessary during the early stages of osteoclast formation. It is generally assumed that during this early stage of osteoclast formation the osteoclast precursor cells proliferate and differentiate into mononuclear preosteoclasts before they fuse to the multinucleated mature osteoclasts [18–20]. Our results indicate that during this initial phase the binding of VCAM-1 to the preosteoclast is one of the necessary factors for osteoclastogenesis to proceed.

The ligand for VCAM-1 on the osteoclast precursor cells could be VLA-4 which belongs to the β_1 integrin subfamily of adhesion molecules. VLA-4 mediated interaction with its ligands has been shown to be essential for several physiologic processes such as lymphopoiesis [12], myogenesis and leukocyte extravasation to the sites of inflammation [21]. In this context it is interesting that VCAM-1 expression has been found on stromal cells of the bone marrow where it mediates binding to lymphocytes precursors [22], osteoclast precursors [23] and to $CD34^{hi}$ hematopoietic stem cells [24] indicating that this adhesion pathway is of general importance during bone marrow stem cell differentiation.

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