CHAPTER 2

Intercellular adhesion molecule-1 clusters during osteoclastogenesis

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Abstract

Adhesion between osteoblasts and osteoclast precursors is established via an interaction involving intercellular adhesion molecule-1 (ICAM-1) on osteoblasts and leukocyte function-associated antigen-1 (LFA-1) on osteoclast precursors. The latter cells also express ICAM-1, but little is known about the expression over time and its possible role during osteoclastogenesis. In the present study we investigated the expression of ICAM-1 on both human osteoblast-like cells and osteoclast precursors in a co-culture. The protein expression on osteoclast precursors strongly increased whereas the osteoblast-like cells became ICAM-1 negative. Interestingly, ICAM-1 on osteoclast precursors manifested as clusters which localized at the baso-lateral membrane. Furthermore, clustered ICAM-1 was associated with F-actin and remained present for several days. Our data suggest that osteoblastic ICAM-1 is mainly involved in the initial adhesion of osteoclast precursors whereas clustered ICAM-1 on osteoclast precursors and its association with F-actin suggest an involvement in cell movement at a later stage.

Introduction

Bone remodeling depends on a tightly balanced regulation of bone formation by osteoblasts and bone resorption by osteoclasts. These resorbing cells are multinucleated, tartrate resistant acid phosphatase (TRACP) positive and formed through fusion of osteoclast precursors. The precursors originate from hematopoietic cells of the monocyte lineage present in the bone marrow and peripheral circulation \(^1,2\) and ultimately fuse to form a syncitium near the bone surface. The involvement of cells of the osteoblast lineage in osteoclast formation is well described \(^3-5\) and adhesion between osteoblasts and osteoclast precursors seems essential for the formation of multinucleated osteoclasts.

Tanaka et al. \(^6\) discerned two functionally different osteoblast populations based on the expression of intercellular adhesion molecule -1 (ICAM-1). ICAM-1-expressing osteoblasts proved to support osteoclast formation, whereas osteoblasts that lack ICAM-1 do not possess that capacity \(^6\). Osteoclast precursors on the other hand express leukocyte function associated antigen-1 (LFA-1), a ligand for ICAM-1. ICAM-1 itself is
also expressed by osteoclast precursors \(^{7,8}\) and its expression increases during osteoclast formation in estrogen deficient mice \(^{7}\). Blocking ICAM-1 in a mono-culture of rat osteoclast precursors decreased the number of osteoclasts formed \(^{8}\), suggesting a role for this molecule in osteoclastogenesis. A shortcoming of most of these studies is the use of osteoclast precursor mono-cultures, which do not resemble *in vivo* osteoclast formation, where osteoblasts are thought to play a crucial role in osteoclast formation. In the present study, we made advantage of human osteoblast-like cells- osteoclast precursor co-cultures, thereby mimicking cellular interactions which appear *in vivo*. Here, following retraction of the osteoblast-like cells the osteoclast precursors migrate to the bone surface and fuse to form osteoclasts \(^{9}\).

This sequence of events has some similarities with diapedesis: attachment of leukocytes to the endothelium is ICAM-1 mediated after which these cells pass the endothelial cell layer \(^{10,11}\). During this process endothelial cells are activated. Their activated state is characterized by clustering of ICAM-1 \(^{12}\) and a co-localization of this molecule with actin filaments. This eventually leads to actin cytoskeletal rearrangements in the endothelial cells.

We hence assessed whether the expression of ICAM-1 and its localization on osteoclast precursors as well as on osteoblast-like cells changes during osteoclast formation, depending on its role in the different stages of this process. We therefore investigated the expression and localization of ICAM-1 on both cell types and analyzed the changes in their actin skeleton during osteoclast formation.

**Materials and methods**

**Cell cultures**

Human osteoblast-like cells were isolated from trabecular bone biopsies from the knee or hip of 6 patients using a previously used procedure \(^{13}\). Bony waste material was obtained with informed consent from patients who underwent knee- or hip surgery at the Department of Orthopedic Surgery at the VU University Medical Centre Amsterdam (VUmc). The biopsies were cut into small pieces (1 mm\(^3\)) and washed extensively with phosphate-buffered saline (PBS; Gibco, Paisley, UK). The fragments were then incubated with 2 mg/ml collagenase II (Sigma, St. Louis, MO) for 2 hours at
37°C to remove all soft tissue on the outer surface of the bone fragments. Next, the bone explants were washed with Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco, Paisley, UK) and cultured in DMEM containing 10% fetal calf serum (FCS; Hyclone, Logan, UT) and 1% antibiotics (100 U/ml penicillin, 100 g/ml streptomycin, and 250 ng/ml amphotericin B [Antibiotic antimycotic solution, Sigma]) in 75 cm² culture flasks. The culture flasks were stored in a humidified atmosphere of 5% CO₂ in air at 37°C. After 2 to 4 weeks, cell monolayers were confluent. The bone fragments were removed and the confluent layers were trypsinized (trypsin; Gibco, Grand Island, NY) and replated. All cells used were expanded for 4 to 7 passages. Osteoblast cultures were established as described previously.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin, Amsterdam, The Netherlands). Buffy coats were diluted 1:1 in Hank’s Buffered Salt Solution (HBSS; Gibco, Paisley, UK). 25 ml of diluted blood was then layered on 15 ml lymphoprep (Axisshield PoCAS, Oslo, Norway) and centrifuged for 30 min at 1,200 g without brake, to create different cell layers. The interphase containing PBMCs was collected and resuspended in HBSS. PBMCs were washed twice in HBSS and finally recovered in DMEM containing 10% fetal calf serum and 1% antibiotics.

**Osteoclastogenesis**

*On plastic.* Osteoclast formation was assessed in co-cultures consisting of a confluent monolayer of human osteoblast-like cells (5x10⁴ cells per well) and 5x10⁵ PBMCs per well seeded on top. The co-culture experiments were performed in 48-well plates (Costar, Cambridge, MA) and the cells were cultured for 3, 9 or 18 days. Culture medium (400 µl/well) was changed twice a week. In addition, PBMCs were cultured in 96 wells plates in the absence of osteoblast-like cells, and in the absence or presence of 25 ng/ml recombinant human M-CSF (R&D Systems, Minneapolis, MN) and 40 ng/ml recombinant human RANKL (PreProtech, Rocky Hill, NJ) for 3, 9 or 18 days.

*On glass.* For confocal microscopy analysis, co-cultures of human osteoblast-like cells and PBMCs were performed on glass slides. For all three substrates, cells were fixed with 4% formaldehyde in PBS for 10 minutes.
TRACP staining
After 3 weeks of culturing, cells were fixed in PBS buffered 4% formaldehyde and stained for tartrate resistant acid phosphatase (TRACP) activity using the leukocyte acid phosphatase kit (Sigma). Nuclei were stained with diamidino-2-phenylindole dihydrochloride (DAPI).

ICAM-1 staining
Fixed cell cultures were washed with PBS and incubated with a mouse anti-human CD54 (ICAM-1) monoclonal antibody (mAb) (1:100, Sanquin, Amsterdam, The Netherlands) or an isotype control IgG1 (1:100) for 1h. After washing thoroughly, an Alexa-488-conjugated goat anti-mouse Ab (1:250, Invitrogen) was added for 1h. Cells were washed and nuclei were stained with diamidino-2-phenylindole dihydrochloride (DAPI) and viewed with a Leica fluorescence microscope (Leica, Wetzlar, Germany) or nuclei were stained with propidium iodide when analysed with confocal microscopy. Image stacks were taken using confocal laser scanning microscopy (Leica).

Actin staining
F-actin was stained according to the method previously described using Alexa-conjugated phalloidin (Molecular Probes, Eugene, OR).

RNA analysis and real-time quantitative PCR
RNA from cultured cells was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. 100 ng RNA was used in the reverse transcriptase reaction which was performed according to the MBI Fermentas cDNA synthesis kit (Vilnius, Lithuania), using both the Oligo(dT)18 and the D(N)6 primers (Table 1).

Real-time PCR was performed on an ABI PRISM 7000 (Applied Biosystems Foster City, CA). The external standard curve used in the PCR reactions was cDNA from the quantitative PCR human reference total RNA (Stratagene, La Jolla, CA), which is composed of RNA obtained from 10 human cell lines.

The reactions were performed with 2 ng cDNA in a total volume of 25 µl containing SYBR Green PCR Master Mix, consisting of SYBR Green 1 Dye, AmpliTaq Gold DNA polymerase, dNTPs, passive reference and buffer (Applied Biosystems) and 300 nM of each primer. After an initial activation step of the AmpliTaq Gold DNA polymerase for 10 minutes at 94 °C, 40
cycles were run of a two step PCR consisting of a denaturation step at 95 °C for 30 seconds and annealing and extension step at 60°C for 1 minute. Subsequently the PCR products were subjected to melting curve analysis to test if any unspecific PCR products were generated.

Porphobilinogen deaminase (PBGD) was used as the housekeeping gene. Expression of this gene was not affected by the experimental conditions. Samples were normalized for the expression of PBGD by calculating the ∆Ct (Ct gene of interest – Ct PBGD) and expression of the different genes is expressed as $2^{-\Delta Ct}$.

Table 1: primers used for quantitative RT - PCR

<table>
<thead>
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<th>Primer</th>
<th>Sequence 5’-&gt;3’</th>
<th>Amplicon length (bp)</th>
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<td>PBGD:</td>
<td></td>
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<tr>
<td>Forward</td>
<td>TgCAgTTTgAAATCATTgCTATgTC</td>
<td>84</td>
</tr>
<tr>
<td>Reverse</td>
<td>AACAggCTTTTCTCTCCAATCTTAgA</td>
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<td>ICAM-1:</td>
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<tr>
<td>Forward</td>
<td>TgAgCAATgTgCAAgAAgATAgC</td>
<td>104</td>
</tr>
<tr>
<td>Reverse</td>
<td>CCCgTTCTTggAgTCCAgTACA</td>
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</table>

Statistical analysis

Linear regression was performed to assess whether there was a significant time dependent increase of ICAM-1 mRNA expression. The level of significance was set at p < 0.05.

Results

Expression of ICAM-1: mRNA and protein

Human PBMCs were co-cultured with human primary osteoblast-like cells for 3 weeks in tissue culture plates. During the first few days of culture PBMCs adhered to osteoblast-like cells. Hereafter the osteoblast-like cells retracted, thus creating a space for the alleged osteoclast precursors to migrate to the surface of the well. Eventually these osteoclast precursors fused and after 3 weeks of culture TRACP-positive multinucleated cells were formed (data not shown).
Assessment of the mRNA expression of ICAM-1 revealed a time-dependent increase (Fig. 1A). ICAM-1 mRNA levels increased 2-fold during osteoclast formation. To study whether this increase of ICAM-1 was reflected by changes in expression of the protein and, if so, whether this was by the osteoblast-like cell or by the osteoclast precursor, immunolocalization was performed at different time points.

At the first time point analyzed (3 days), ICAM-1 was expressed on both cell types (Fig. 1B). In line with data previously described by Tanaka et al. \(^6\), ICAM-1-positive and ICAM-1-negative osteoblast-like cells were present. However, after 9 days of co-culture almost all osteoblast-like cells were ICAM-1-negative whereas the osteoclast precursors highly expressed the molecule. Not only the expression level, but also the expression pattern of ICAM-1 on osteoclast precursors changed time-dependently. During the first days, in the early stage of osteoclast formation, ICAM-1 was uniformly distributed at the membrane while after 9 days of co-culture ICAM-1 showed a dot-like or clustered pattern. Moreover, the morphology of the precursors changed during the culture period. The initially round osteoclast precursor became elongated. After 18 days of co-culture, the osteoclast precursor was round again and it highly expressed the protein ICAM-1 which was now mostly seen in a uniform pattern. Multinucleated osteoclast-like cells formed at the end of the co-culture period were either ICAM-1-negative or positive. At this time point ICAM-1 was uniformly distributed (Fig. 1B).

Quantifying the number of ICAM-1-positive cells at the end of the culture period revealed that 66% of the TRACP+ cells also expressed ICAM-1. These double positive cells represented 24% of the total number of cells analyzed.
Figure 1. ICAM-1 increases in a co-culture of osteoblasts and osteoclast precursors. Human PBMCs were cultured with primary osteoblast-like cells and ICAM-1 expression was analyzed. A. ICAM-1 mRNA expression after 3, 9 and 18 days of co-culture. The values indicate the mean expression and SEM in co-cultures of PBMCs and osteoblast-like cells from 6 patients. A linear regression was performed and showed a time dependent increase of ICAM-1 mRNA expression. The fit line (−), the 95% confidence interval (−−), the p-value (0.02) and the R2- value (0.03) are depicted. B. ICAM-1 protein expression after 3, 9 and 18 days of co-culturing. Green staining indicates the presence of ICAM-1 on osteoblast-like cells (OB, closed arrows) and osteoclast precursors (OCp, open arrows). Nuclei in blue were visualized with DAPI. Images were taken at 200x magnification. For color figure see p. 154.

In order to obtain a better insight in the localization of the ICAM-1-clusters found on osteoclast precursors, we studied the spatial localization of this molecule, using confocal microscopy. Based on a preliminary study in which we investigated the ICAM-1 expression over time in more detail (data not shown), we stained ICAM-1 in a co-culture of osteoblast-like cells and osteoclast precursors, after 15 days of culture. At this time point ICAM-1 was still clustered and osteoclast precursors were migrating. Confocal imaging showed clustering of ICAM-1 on osteoclast precursors and a limited expression on osteoblast-like cells (Fig. 2). This is in line with the findings described above. The confocal cross-sections further revealed that ICAM-1 clusters were exclusively seen on the baso-lateral membrane of the osteoclast precursor, an area different from the contact side between osteoclast precursors and osteoblast-like cells. This particular localization
suggests that this molecule is probably not directly involved in the adhesion to osteoblast-like cells at this stage of osteoclast formation.

**Figure 2.** ICAM-1 in a co-culture reveals a baso-lateral localization of the molecule on osteoclast precursors. The confocal image shows a top view (center) of a co-culture of osteoblast-like cells (OB) and osteoclast precursors (OCp) after a 15 days culture period. The horizontal and vertical cross sections are depicted on the bottom and the right of the top view, respectively. The green staining indicates the presence of ICAM-1 and the nuclei are visualized in red. ICAM-1 is expressed exclusively on baso-lateral membranes of osteoclast precursors. For color figure see p.154.

**Co-localization with F-Actin**

Yang et al.\textsuperscript{11} have shown that clustered ICAM-1 on activated endothelial cells was associated with the actin filaments of the cytoskeleton. Based on these findings and the clustering of ICAM-1 on osteoclast precursors, we next investigated the localization of F-actin in osteoclast precursors during the different time points in the process of osteoclast formation. Fig. 3 shows double staining of F-actin and ICAM-1 in a co-culture of osteoblast-like cells and osteoclast precursors. At 3 days, ICAM-1 did not co-localize with filamentous actin. Evident co-localization was seen after 9 days. Near the end of the culture, after 18 days, co-localization of ICAM-1 and F-actin was only observed in a few cells.
Figure 3. ICAM-1 co-localizes with filamentous actin during ICAM-1 clustering. The localization of ICAM-1 changes from a crescent (3 days, left panel) to a clustered (9 days, middle panel) and finally to a diffuse (18 days, right panel) localization. ICAM-1 co-localizes with filamentous actin at 9 days. Co-localization is not present at 3 days and disappears at 18 days of co-culture. Images were taken at 200x magnification. For color figure see p. 155.

**ICAM-1 in a mono-culture of osteoclast precursors**

To study whether the presence of osteoblast-like cells is a prerequisite for ICAM-1 expression and/or clustering on osteoclast precursors, we next employed an osteoclastogenesis assay in the absence of osteoblast-like cells. We assessed osteoclast formation in a mono-culture of PBMCs cultured with or without M-CSF and RANKL. In both mono-cultures, a similar pattern of ICAM-1 distribution was seen and this pattern was comparable to the one seen in the co-culture system: ICAM-1 clusters were absent after 3 days and present at 9 and 18 days. Clustering peaked at 12 days, whereafter a gradual decline in the percentage ICAM-1 cluster positive osteoclast precursors was observed (Fig. 4A). Addition of osteoclast-related cytokines M-CSF and RANKL resulted in approximately 20% more ICAM-1 cluster positive cells from day 12 till 21 (Fig. 4B).
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A. Protein staining of ICAM-1 (green) shows clustering of ICAM-1 (arrows) after 9 days of culture in the presence (upper panel) and absence (lower panel) of M-CSF and RANKL. B. The number of PBMCs that show ICAM-1 clusters, indicated as the percentage of the total number of cells. For each culture condition, three images were analyzed. Images were taken at 200x magnification. For color figure see p. 155.

Discussion

Adhesion of osteoclast precursors to osteoblasts is an essential step in the process of osteoclast formation and is regulated via an interaction of ICAM-1 on the osteoblast and LFA-1 on the osteoclast precursor. The latter cell type also expresses ICAM-1 and blocking this molecule on osteoclast
precursors in a mono-culture or co-culture leads to a reduced number of osteoclasts, suggesting a role for ICAM-1 on osteoclast precursors in osteoclastogenesis. In the present study we show that the mRNA expression of ICAM-1 in a co-culture of osteoblast-like cells and osteoclast precursors increases during osteoclast formation. Analysis of the expression of the protein revealed a decrease over time on osteoblast-like cells whereas ICAM-1 increases on osteoclast precursors. This suggests that the role of ICAM-1 on the two cell types changes during the process of osteoclast formation. ICAM-1 on osteoblast-like cells is required in an early stage of osteoclast formation, when these cells bind osteoclast precursors through an ICAM-1:LFA-1 interaction. At later stages of osteoclastogenesis expression of ICAM-1 is more prominent on osteoclast precursors, while osteoblast-like cells hardly express the molecule. This is in line with a study of Kurachi et al. in which they analyzed ICAM-1 in a co-culture of rat spleen cells and mouse bone marrow derived clonal stromal cells. They separately blocked ICAM-1 on the spleen cells and stromal cells using an anti-rat mAB and anti-mouse mAB, respectively. When ICAM-1 on the stromal cells was blocked, a reduction of TRACP-activity was found when the antibody was applied during the first days of co-culture. On the other hand, blocking ICAM-1 on the spleen cells led to a reduction in TRACP-activity only when the antibody was added at the end of the culture. These observations therefore also suggested that ICAM-1 on the osteoclast precursor cells is involved in final stages of osteoclast formation such as fusion, whereas osteoblastic ICAM-1 is primarily needed in an early stage.

After adhesion, osteoclast precursors migrate to the bone surface and fuse to form multinucleated osteoclasts. It is suggested that the fusion of osteoclast precursors is ICAM-1-mediated. However, a specific function of this molecule during fusion has not been described. Our results showed a change in ICAM-1 distribution on osteoclast precursors over time. During the initial days of culturing, ICAM-1 was uniformly distributed on the membrane of the osteoclast precursor whereas after 9 days the molecule was clustered and more distributed in a patch-like pattern. This phenomenon of ICAM-1 clustering has not been noted before in the process of osteoclast formation. Yet, it is well described in endothelial cells involved in diapedesis. Attachment of leukocytes to the endothelium is ICAM-1-mediated and is followed by migration and penetration of these cells through the endothelial cell layer. It is thought that clustered ICAM-1 on endothelial cells
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indicates an activated stage of these cells \(^17,20\). Taken together, this suggests that osteoclast precursors become activated during osteoclast formation. Also analogous to activated endothelial cells we further showed a dominant co-localization of clustered ICAM-1 and F-actin on osteoclast precursors at intermediate time points. This was not present in an early stage, or when multinucleated osteoclasts were formed \(^18,21,22\). Interestingly, the clusters on osteoclast precursors were not present in the very first days of culturing. After 3 days the first ICAM-1 clusters appeared while after 18 days almost all clusters had disappeared. In the sequence of events, this rather lengthy period of time corresponds to the time frame between osteoblast-osteoclast precursor cell-cell adhesion, migration of osteoclast precursors away from the osteoblast and the ultimate formation of a syncitium.

Our observations further indicate that the initially round osteoclast precursors become elongated during osteoclast differentiation. The cytoskeletal rearrangements occur after adhesion and the elongated cells become round again prior to fusion. Compared to round cells, ICAM-1 expression appeared predominantly as clusters in elongated osteoclast precursors. At this stage, the cells need to migrate to the bone surface to fuse with other osteoclast precursors. This could indicate that clustering of ICAM-1 on osteoclast precursors and the changes in their cytoskeleton are associated with cell movement, required for migration, facilitating the encounter of fellow fusing cells. Importantly, also retracting osteoblast-like cells need to rearrange their cytoskeleton, but during these stages no ICAM-1 expression was observed.

To investigate if clustering of ICAM-1 and the possible activation of osteoclast precursors is influenced by the absence of osteoblast-like cells, we used a mono-culture of human peripheral blood mononuclear cells and stimulated the cells with or without M-CSF and RANKL, cytokines known to be required for osteoclast formation \textit{in vivo}. When using PBMCs from healthy volunteers, we have recently shown that multinucleated cells can be obtained even without addition of M-CSF and RANKL. However, these cytokines were pivotal for achieving bone resorbing activity \(^23\). Here, we show that the changes in ICAM-1 expression and localization were comparable to the results found in a co-culture. The clusters of ICAM-1, however, were present in approximately 20% more cells when M-CSF and RANKL were added. This suggests that the clustering of ICAM-1 is not
dependent on the presence of osteoblast-like cells, but is positively influenced by the osteoclast-related cytokines produced by these cells. This study demonstrates transient changes in expression of the adhesion molecule ICAM-1 during the process of osteoclast formation. The initial expression and its subsequent decreased expression on osteoblast-like cells suggests that this molecule on this cell type is mainly involved in adhesion whereas the shift in location on the osteoclast precursors together with the co-localization of clustered ICAM-1 and F-actin strongly suggests a novel role for ICAM-1, other than adhesion. We surmise that clustered ICAM-1 on osteoclast precursors leads to intracellular signaling and eventually to cytoskeletal re-arrangements in order to activate the cell for migration.
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References


