Increase in ALK1/ALK5 Ratio as a Cause for Elevated MMP-13 Expression in Osteoarthritis in Humans and Mice

Esmeralda N. Blaney Davidson, Dennis F. G. Remst, Elly L. Vitters, Henk M. van Beuningen, Arjen B. Blom, Marie-Jose Goumans, Wim B. van den Berg and Peter M. van der Kraan

*J Immunol* 2009; 182:7937-7945; doi: 10.4049/jimmunol.0803991
http://www.jimmunol.org/content/182/12/7937

References

This article cites 39 articles, 11 of which you can access for free at:
http://www.jimmunol.org/content/182/12/7937.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

Errata

An erratum has been published regarding this article. Please see next page or:
/content/185/4/2629.full.pdf
Increase in ALK1/ALK5 Ratio as a Cause for Elevated MMP-13 Expression in Osteoarthritis in Humans and Mice

Esmeralda N. Blaney Davidson,†* Dennis F. G. Remst,* Elly L. Vitters,* Henk M. van Beuningen,* Arjen B. Blom,* Marie-Jose Goumans,† Wim B. van den Berg,* and Peter M. van der Kraan*

During osteoarthritis (OA) chondrocytes show deviant behavior resembling terminal differentiation of growth-plate chondrocytes, characterized by elevated MMP-13 expression. The latter is also a hallmark for OA. TGF-β is generally thought to be a protective factor for cartilage, but it has also displayed deleterious effects in some studies. Recently, it was shown that besides signaling via the ALK5 (activin-like kinase 5) receptor, TGF-β can also signal via ALK1, thereby activating Smad1/5/8 instead of Smad2/3. The Smad1/5/8 route can induce chondrocyte terminal differentiation. Murine chondrocytes stimulated with TGF-β activated the ALK5 receptor/Smad2/3 route as well as the ALK1/Smad1/5/8 route. In cartilage of mouse models for aging and OA, ALK5 expression decreased much more than ALK1. Thus, the ALK1/ALK5 ratio increased, which was associated with changes in the respective downstream markers: an increased Id-1 (inhibitor of DNA binding-1)/PAI-1 (plasminogen activator inhibitor-1) ratio. Transfection of chondrocytes with adenosine overexpressing constitutive active ALK1 increased MMP-13 expression, while small interfering RNA against ALK1 decreased MMP-13 expression to nondetectable levels. Adenovirus overexpressing constitutive active ALK5 transfection increased aggrecan expression, whereas small interfering RNA against ALK5 resulted in increased MMP-13 expression. Moreover, in human OA cartilage ALK1 was highly correlated with MMP-13 expression, whereas ALK5 correlated with aggrecan and collagen type II expression, important for healthy cartilage. Collectively, we show an age-related shift in ALK1/ALK5 ratio in murine cartilage and a strong correlation between ALK1 and MMP-13 expression in human cartilage. A change in balance between ALK5 and ALK1 receptors in chondrocytes caused changes in MMP-13 expression, thereby causing an OA-like phenotype. Our data suggest that dominant ALK1 signaling results in deviant chondrocyte behavior, thereby contributing to age-related cartilage destruction and OA. The Journal of Immunology, 2009, 182: 7937–7945.
now. Therefore, we have studied ALK1 and ALK5 expression in articular cartilage in aging mice, experimental models of OA, and human OA. Moreover, we have examined the functional consequences of dominant ALK1 or ALK5 signaling in chondrocytes, revealing that a shift in balance toward ALK1 induces an OA phenotypic change, thereby providing a new view on TGF-β signaling during aging and OA.

Materials and Methods

Cell culture

Chondrocytes were obtained from mouse cartilage by digestion with collagenase. They were immortalized by transduction with SV40 large T Ag and subsequently cloned by minimal dilution as previously described (22). All chondrocyte cell lines had both ALK5 and ALK1 expression. The chondrocyte cell line (H1) was cultured in 10^5 cells per well in 24-well plates in DMEM/HAM’s F12 (1:1) with 10% FCS and penicillin and streptomycin.

The H1 chondrocytes were stimulated with TGF-β (1 ng/ml) under serum-free conditions to evaluate functional TGF-β signaling via ALK5 or ALK1. This experiment was repeated in bovine primary chondrocytes to ensure that the chondrocyte cell line responded similarly to primary cells. The primary bovine chondrocytes were isolated from bovine metacarpophalangeal joint as previously described (23).

For the different Ags, the number of positive articular chondrocytes in the tibia was determined by a blinded observer using the Qwin image analysis. Animals were considered to have hyaline cartilage degeneration, the signature pathologic feature of OA, if they had cartilage matrix degradation and/or regeneration/repair, chondrocyte death, chondrocyte replication, chondrocyte proliferation, cartilage lesions, bone lesion soft tissue laxity, or a combination of lesions (28).

Animals

Male C57BL/6N mice aged 1 year (n = 7) and 2 years (n = 10) were used for comparison of young vs old mice. C57BL/6N mice aged 10 wk were used to induce OA by transaction of the medial meniscus (n = 10) (destabilization of the medial meniscus (DMM) model). In this surgical mouse model the anterior attachment of the medial meniscus to the tibia is transected, as previously described by Glisson et al. (25). Mice were sacrificed 8 wk after surgery.

Histology

Knee joints of sacrificed mice were isolated for histology. OA was confirmed by histological evaluation. Animals were considered to have hyaline cartilage degeneration, the signature pathologic feature of OA, if they had cartilage matrix degradation and/or regeneration/repair, chondrocyte death, chondrocyte replication, chondrocyte proliferation, cartilage lesions, bone lesion soft tissue laxity, or a combination of lesions (28).

Histology

Knee joints were fixed in phosphate-buffered formalin for 7 days. They were dehydrated using an automated tissue-processing apparatus (Tissue-Tek VIP; Sakura) and embedded in paraffin. Tissue sections of 7 μm were prepared. Immunohistochemistry was performed as previously described (21).

Western blot

For Western blot analyses each sample was measured for protein concentration, after which 20 μg of each protein sample was loaded on a SDS 7.5% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (Hybond-P; Amersham Pharmacia Biotech) using the IBlot system (Invitrogen). The membrane was incubated with Abs against Smad1/5/8 (1/1000) and Smad2P (1/1000) (Cell Signaling Technology) overnight at 4°C. The secondary HRP-conjugated Ab was goat-anti-rabbit (1/1500) (Dako), and the membrane was developed using the ECL Plus Western blotting detection system (GE Healthcare).

Animals

Male C57BL/6N mice aged 1 year (n = 7) and 2 years (n = 10) were used for comparison of young vs old mice. C57BL/6N mice aged 10 wk were used to induce OA by transaction of the medial meniscus (n = 10) (destabilization of the medial meniscus (DMM) model). In this surgical mouse model the anterior attachment of the medial meniscus to the tibia is transected, as previously described by GLisson et al. (25). Mice were sacrificed 8 wk after surgery.

Histology

Knee joints of sacrificed mice were isolated for histology. OA was confirmed by histological evaluation. Animals were considered to have hyaline cartilage degeneration, the signature pathologic feature of OA, if they had cartilage matrix degradation and/or regeneration/repair, chondrocyte death, chondrocyte replication, chondrocyte proliferation, cartilage lesions, bone lesion soft tissue laxity, or a combination of lesions (28).

Animals were kept in filter top cages with woodchip bedding under standard pathogen-free conditions. They were fed a standard diet with tap water ad libitum. The Animal Ethics Committee of the Radboud University Nijmegen approved all animal procedures.

Histology

Knee joints were fixed in phosphate-buffered formalin for 7 days. They were dehydrated using an automated tissue-processing apparatus (Tissue-Tek VIP; Sakura) and embedded in paraffin. Tissue sections of 7 μm were prepared.

Immunohistochemistry was performed as previously described (21). Specific primary Abs against ALK1 (1/1000), ALK5 (1/100), Id-1 (inhibitor of DNA binding-1) (1/1000), and PAI-1 (plasminogen activator inhibitor-1) (1/1000) were incubated overnight at 4°C (purchased from Santa Cruz Biotechnology). As a negative control, the primary Ab was replaced with goat or rabbit IgGs. A biotin-streptavidin detection system was used according to the manufacturer’s protocol (Vector Laboratories). Bound complexes were visualized via reaction with 3,3'-diaminobenzidine (Sigma-Aldrich) and H₂O₂ resulting in a brown precipitate. Sections were counterstained with hematoxylin and mounted with Permount.

Quantitative PCR

Quantitative PCR was performed as previously described on an ABI Prism 7000 sequence detection system (Applied Biosystems) (24). Efficiencies (E) for all primer sets were determined (Table I) using a standard curve of five serial cDNA dilutions in water in duplicate. Primers were accepted if the deviation from the slope of the standard curve was <0.3 compared with the slope of the GAPDH standard curve and if the melting curve showed only one product.

Western blot

For Western blot analyses each sample was measured for protein concentration, after which 20 μg of each protein sample was loaded on a SDS 7.5% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (Hybond-P; Amersham Pharmacia Biotech) using the IBlot system (Invitrogen). The membrane was incubated with Abs against Smad1/5/8 (1/1000) and Smad2P (1/1000) (Cell Signaling Technology) overnight at 4°C. The secondary HRP-conjugated Ab was goat-anti-rabbit (1/1500) (Dako), and the membrane was developed using the ECL Plus Western blotting detection system (GE Healthcare).

Animals

Male C57BL/6N mice aged 1 year (n = 7) and 2 years (n = 10) were used for comparison of young vs old mice. C57BL/6N mice aged 10 wk were used to induce OA by transaction of the medial meniscus (n = 10) (destabilization of the medial meniscus (DMM) model). In this surgical mouse model the anterior attachment of the medial meniscus to the tibia is transected, as previously described by GLisson et al. (25). Mice were sacrificed 8 wk after surgery.

Histology

Knee joints of sacrificed mice were isolated for histology. OA was confirmed by histological evaluation. Animals were considered to have hyaline cartilage degeneration, the signature pathologic feature of OA, if they had cartilage matrix degradation and/or regeneration/repair, chondrocyte death, chondrocyte replication, chondrocyte proliferation, cartilage lesions, bone lesion soft tissue laxity, or a combination of lesions (28).

Animals were kept in filter top cages with woodchip bedding under standard pathogen-free conditions. They were fed a standard diet with tap water ad libitum. The Animal Ethics Committee of the Radboud University Nijmegen approved all animal procedures.

Histology

Knee joints were fixed in phosphate-buffered formalin for 7 days. They were dehydrated using an automated tissue-processing apparatus (Tissue-Tek VIP; Sakura) and embedded in paraffin. Tissue sections of 7 μm were prepared.

Immunohistochemistry was performed as previously described (21). Specific primary Abs against ALK1 (1/1000), ALK5 (1/100), Id-1 (inhibitor of DNA binding-1) (1/1000), and PAI-1 (plasminogen activator inhibitor-1) (1/1000) were incubated overnight at 4°C (purchased from Santa Cruz Biotechnology). As a negative control, the primary Ab was replaced with goat or rabbit IgGs. A biotin-streptavidin detection system was used according to the manufacturer’s protocol (Vector Laboratories). Bound complexes were visualized via reaction with 3,3'-diaminobenzidine (Sigma-Aldrich) and H₂O₂ resulting in a brown precipitate. Sections were counterstained with hematoxylin and mounted with Permount.

Table I. Primers used for quantitative PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>R²</th>
<th>E²</th>
<th>Forward Primer (5’→3’)</th>
<th>Reverse Primer (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>0.997</td>
<td>2.05</td>
<td>GGCAAAATCCACGGGCAACA</td>
<td>GTTATGGCGTGCTGCTTGGCTTGC</td>
</tr>
<tr>
<td>ALK1</td>
<td>0.999</td>
<td>1.90</td>
<td>ACCATTGCTGATGGTCCGACTCTG</td>
<td>GTCATGGGACCACTACATC</td>
</tr>
<tr>
<td>ALK5</td>
<td>0.999</td>
<td>2.02</td>
<td>AGCGGCTGCTGCCATCCT</td>
<td>AGCAATGCTGGTGCTTGGAGT</td>
</tr>
<tr>
<td>Collagen I</td>
<td>0.992</td>
<td>2.15</td>
<td>TCCACTCCACGCTATGGGAGA</td>
<td>GACCTAGGCTGTTGGGAGA</td>
</tr>
<tr>
<td>Aggrocen</td>
<td>0.992</td>
<td>2.15</td>
<td>TCCACTCCACGCTATGGGAGA</td>
<td>GACCTAGGCTGTTGGGAGA</td>
</tr>
<tr>
<td>MMP-13</td>
<td>0.992</td>
<td>1.93</td>
<td>ACCTGGTGTGTTGGCACGACTAATTGG</td>
<td>TCTCAGGATCTCGGACCAGAGT</td>
</tr>
<tr>
<td>Human</td>
<td>0.999</td>
<td>1.92</td>
<td>ACTCTCCTTTTGGCTGCGGAC</td>
<td>TCCCAGGACATGGTCTGACG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>0.999</td>
<td>2.02</td>
<td>GACTTCAAGAGCCGCAATGGG</td>
<td>GTCGCGGAGTGGCAAC</td>
</tr>
<tr>
<td>ALK5</td>
<td>0.995</td>
<td>2.05</td>
<td>CGACGGCTGCTGCCATCCT</td>
<td>CCCACTGTCACGACAAAGTAAATTG</td>
</tr>
<tr>
<td>Collagen I</td>
<td>0.995</td>
<td>2.00</td>
<td>CACGTACACTGCCCTGAGAA</td>
<td>CAAACAGCTGCTGCCCCACCT</td>
</tr>
<tr>
<td>Aggrocen</td>
<td>0.997</td>
<td>2.01</td>
<td>GCCTGGGCGCCAATTGAC</td>
<td>ATGGGACACAGATGGCCCTTTACC</td>
</tr>
<tr>
<td>MMP-13</td>
<td>0.998</td>
<td>1.95</td>
<td>ATTAAGGAGACATGCGGCACT</td>
<td>CCGAGGAGAAAGACAGT</td>
</tr>
</tbody>
</table>

*Efficiency.
Samples of human cartilage were obtained from patients undergoing knee or hip replacement surgery. The cartilage was instantly frozen in liquid nitrogen and crushed using a dismembrator. The crushed material was dissolved in RLT buffer supplied with the RNeasy Mini kit (Qiagen). Samples were treated with proteinase K, and subsequently RNA was isolated further with the RNeasy Mini kit according to the manufacturer’s protocol, after which an RT-PCR was performed followed by a quantitative PCR.

The Medical Ethics Committee of Radboud University Nijmegen Medical Centre approved the study protocol.

**Statistical analysis**

Results were analyzed with Student’s t test and considered significant if the p value was <0.05. Correlations were tested with Pearson’s correlation and considered significant if the p value was <0.05.

**Results**

Chondrocytes signal both via ALK5 and ALK1

Alternative signaling of TGF-β via ALK1 has been shown in endothelial cells, but it was not known whether chondrocytes have the ability to signal via both type I TGF-β receptors as well. Therefore, expression of both type I TGF-β receptors, ALK1 and ALK5, on chondrocytes was evaluated by quantitative RT-PCR. This showed that both ALK1 and ALK5 were clearly expressed in murine cartilage, murine chondrocyte cell lines, bovine cartilage, and human cartilage (data not shown). Stimulation of H1 chondrocytes with TGF-β led to both Smad1/5/8 and Smad2/3 phosphorylation within 15 min, which lasted for at least 5 h (Fig. 1A). The enhanced phosphorylation levels vanished after 24 h (Fig. 1A). To determine if, similar to the chondrocyte cell line, primary chondrocytes were also able to signal via both

[FIGURE 1. TGF-β signals through ALK1 and ALK5 in chondrocytes. A, Chondrocytes, both the murine H1 cell lines as the primary bovine chondrocytes, are able to signal via both ALK1 and ALK5 upon TGF-β stimulation, thereby displaying phosphorylation of Smad1/5/8 and Smad2, respectively. B, Chondrocytes (H1 cell line) were transduced with Ad-caALK1 or Ad-caALK5 after which Western blot was performed for Smad1/5/8P and Smad2P. Adenoviral overexpression of constitutive ALK5 leads to Smad2 phosphorylation, while constitutive ALK1 results in Smad1/5/8 phosphorylation.]

**FIGURE 2.** The ALK1/ALK5 ratio increases with age. Murine knee joints of mice aged 1 or 2 years were isolated and prepared for paraffin sections in order to perform immunohistochemistry for ALK1 and ALK5. The number of cells staining positive in the tibia were measured with a computerized imaging system and corrected for the total number of cells in H&E-stained sections. The number of positive cells for ALK5 is strongly reduced with age (A), whereas the reduction in ALK1 staining is not significant (B). This results in a shift in the ALK1/ALK5 ratio with age favoring the ALK1 side (C). Representative sections are displayed in D. *, p < 0.05; **, p < 0.005; ***, p < 0.001.

Human cartilage samples

Samples of human cartilage were obtained from patients undergoing knee or hip replacement surgery. The cartilage was instantly frozen in liquid nitrogen and crushed using a dismembrator. The crushed material was dissolved in RLT buffer supplied with the RNeasy Mini kit (Qiagen). Samples were treated with proteinase K, and subsequently RNA was isolated further with the RNeasy Mini kit according to the manufacturer’s protocol, after which an RT-PCR was performed followed by a quantitative PCR.

The Medical Ethics Committee of Radboud University Nijmegen Medical Centre approved the study protocol.

**Statistical analysis**

Results were analyzed with Student’s t test and considered significant if the p value was <0.05. Correlations were tested with Pearson’s correlation and considered significant if the p value was <0.05.

**Results**

Chondrocytes signal both via ALK5 and ALK1

Alternative signaling of TGF-β via ALK1 has been shown in endothelial cells, but it was not known whether chondrocytes have the ability to signal via both type I TGF-β receptors as well. Therefore, expression of both type I TGF-β receptors, ALK1 and ALK5, on chondrocytes was evaluated by quantitative RT-PCR. This showed that both ALK1 and ALK5 were clearly expressed in murine cartilage, murine chondrocyte cell lines, bovine cartilage, and human cartilage (data not shown). Stimulation of H1 chondrocytes with TGF-β led to both Smad1/5/8 and Smad2/3 phosphorylation within 15 min, which lasted for at least 5 h (Fig. 1A). The enhanced phosphorylation levels vanished after 24 h (Fig. 1A). To determine if, similar to the chondrocyte cell line, primary chondrocytes were also able to signal via both

[FIGURE 1. TGF-β signals through ALK1 and ALK5 in chondrocytes. A, Chondrocytes, both the murine H1 cell lines as the primary bovine chondrocytes, are able to signal via both ALK1 and ALK5 upon TGF-β stimulation, thereby displaying phosphorylation of Smad1/5/8 and Smad2, respectively. B, Chondrocytes (H1 cell line) were transduced with Ad-caALK1 or Ad-caALK5 after which Western blot was performed for Smad1/5/8P and Smad2P. Adenoviral overexpression of constitutive ALK5 leads to Smad2 phosphorylation, while constitutive ALK1 results in Smad1/5/8 phosphorylation.]

**FIGURE 2.** The ALK1/ALK5 ratio increases with age. Murine knee joints of mice aged 1 or 2 years were isolated and prepared for paraffin sections in order to perform immunohistochemistry for ALK1 and ALK5. The number of cells staining positive in the tibia were measured with a computerized imaging system and corrected for the total number of cells in H&E-stained sections. The number of positive cells for ALK5 is strongly reduced with age (A), whereas the reduction in ALK1 staining is not significant (B). This results in a shift in the ALK1/ALK5 ratio with age favoring the ALK1 side (C). Representative sections are displayed in D. *, p < 0.05; **, p < 0.005; ***, p < 0.001.
routes in response to TGF-β, the experiment was repeated with primary bovine chondrocytes. These chondrocytes also responded to TGF-β by phosphorylation of Smad1/5/8 and Smad2. The bovine primary chondrocytes had a slower response on Smad1/5/8 phosphorylation (Fig. 1A). These data show that TGF-β is not only able to signal via the Smad2/3 pathway, but also via the Smad1/5/8 route in chondrocytes.

To assess whether activation of the ALK1 pathway leads to the expected Smad1/5/8 phosphorylation and ALK5 to Smad2/3 phosphorylation, we transduced the H1 chondrocytes with Ad-caALK1, Ad-caALK5, or Ad-LacZ as a control. The ΔΔC_T values of transfected samples vs nontransfected controls was 10.99 for ALK1 and 11.3 for ALK5, and thus transfection led to a comparable increase in mRNA expression levels. It was clear that transfection with Ad-ALK1 specifically led to phosphorylation of Smad1/5/8P, whereas ALK5 resulted in Smad2 phosphorylation (Fig. 1B).

Reduction in ALK5 expression is much stronger than reduction in ALK1 in aging mice

Our group has previously demonstrated that aging was accompanied by a decrease in signaling via ALK5 in murine models for OA (20, 21) To investigate potential changes in ALK1 expression with age, we stained sections of knee joints of mice aged 1 year or 2 years immunohistochemically for ALK5 and ALK1. The number of both ALK1 and ALK5 immunopositive cells decreased with age (Fig. 2, A and B). For ALK1, the number of cells staining positive was reduced, with 15% in the medial tibial cartilage and 17% in the lateral tibial cartilage, but this was not significant. However, the number of ALK5-positive cells decreased, with even 80% and 70%, respectively. Thus, the drop in ALK5 expression is much more pronounced than the drop in ALK1 expression with age, hence altering the receptor balance with age (Fig. 2C).

Increased ALK1/ALK5 ratio in focal cartilage degeneration in instability-induced OA: the DMM model

In the DMM model, the ligament attaching the medial meniscus to the tibia is transected. This resulted in joint instability and OA development. As expected, the initial changes could be seen in the cartilage on the medial side of the tibia. The number of ALK1- and ALK5-positive cells was measured in the tibial cartilage both on the medial and the lateral side of the joint. No change in ALK5 and ALK1 expression was found on the lateral side of the joint, but a drastic decrease in both ALK1- and ALK5-positive cells in the medial tibial plateau was observed (Fig. 3). The number of ALK5-positive cells had decreased 89% compared with a 68% decrease of cells positive for ALK1 (Fig. 3). This emphasizes that in experimental OA the number of cells expressing ALK5 declines more rapidly than the number of cells expressing ALK1, thus increasing the ALK1/ALK5 ratio.
The ALK1/ALK5 ratio increases with OA progression in spontaneous OA: STR/ort mice

STR/ort mice spontaneously develop OA at an early age. In STR/ort mice 2–3 mo old, initial changes can be seen on the medial tibia. Eighty percent of male mice have OA lesions by 6 mo of age. To assess the type I TGF-β receptor expression during spontaneous OA, changes in the numbers of cells staining positive for ALK1 and ALK5 in time were analyzed. This revealed a decrease in the number of positive cells for both ALK1 and ALK5 with age, and thus with progression of OA. The ALK1/ALK5 ratio clearly increased with progression of OA (Fig. 4). Where the number of cells staining positive for ALK5 was equal to ALK1 in the lateral tibia at 3 mo, the number of cells staining positive for ALK5 in the medial tibia was only 1% compared with 26% of the cells staining positive for ALK1. Strikingly, STR/ort mice develop OA first on the medial plateau. This is also the plateau that shows the first signs of decrease in ALK5 expression. These data again demonstrate that with progression of OA, the ALK1/ALK5 ratio increases (Fig. 4C).

High Id-1/PAI-1 ratio in aging STR/ort mice

Changes in receptor balance do not necessarily imply downstream alterations. Therefore, we investigated the changes in the corresponding downstream markers PAI-1 (downstream ALK5) (19) and Id-1 (downstream ALK1) (29) in a model for primary OA using STR/ort mice.

The Id-1/PAI-1 ratio was already very high at 3 mo of age in the medial tibial cartilage, where OA is first found. On the lateral side of the tibia this ratio was much lower at an age of 3 mo. However, both the medial and the tibial side of the tibia showed a pronounced increase in the Id-1/PAI-1 ratio during aging from 3 mo to 1 year. Thus, not only did we find altered receptor expression during OA progression, but also alterations in the downstream markers of the corresponding signaling pathways (Fig. 5).

ALK1 expression is significantly correlated with MMP-13 expression in human OA cartilage

We found an increase in ALK1/ALK5 ratio with OA progression. This would suggest a correlation between ALK1 and deleterious effects in OA cartilage. To investigate this, cartilage samples of 20 OA patients undergoing joint replacement surgery were obtained for RNA isolation. The mRNA expression levels of ALK5, ALK1, essential matrix molecules collagen type II and aggrecan, and the most important degrading enzyme of collagen type II and marker...
ALK1 is a potential cause of OA-like changes, chondrocytes were transduced with Ad-caALK5 and Ad-caALK1. Overexpression of caALK5 resulted in a significantly increased expression of aggrecan mRNA, indicating an anabolic response (Fig. 7A). Overexpression of caALK1 also showed a trend toward up-regulation of collagen type II and aggrecan, but this was not significant. However, overexpression of caALK1 resulted in a strikingly different pattern on MMP-13 expression. ALK1 signaling induced a significant increase in MMP-13 mRNA levels. Thus, caALK1 overexpression resulted in elevated expression of the chondrocyte terminal differentiation marker and marker for OA, MMP-13, while caALK5 induces elevated aggrecan mRNA levels and had no significant effect on MMP-13 mRNA levels.

### Knockdown of ALK5 increases MMP-13 mRNA levels

As we found significant changes in the ALK1/ALK5 ratio in vivo and a clear association between higher ALK1 and MMP-13, the most crucial question that remained was whether this reduction in ALK5 and thus a shift in ALK1/ALK5 ratio that we found with age could actually induce elevation of MMP-13. Therefore, chondrocytes were transfected with a plasmid containing shRNA against ALK5. This resulted in reduced levels of ALK5 expression (reduction of 1.8 cycles) and induced increased levels of MMP-13 mRNA compared with the negative plasmid control (5.7 cycles). Transfection of chondrocytes with shRNA for ALK1 resulted in nondetectable levels ALK1 and nondetectable levels of MMP-13 and thus a reduction in MMP-13. No changes were found in the expression of aggrecan or collagen type II. Although Ad-caALK5 significantly increased aggrecan expression, the difference between Ad-caALK5- and Ad-caALK1-induced aggrecan expression was not significant. Thus, it is not expected that inhibition of only one receptor will result in significant changes in aggrecan expression. These findings show that the change in balance between ALK1 and ALK5 that was found with age and even more so during OA will result in elevated MMP-13 levels in chondrocytes downstream, which is a hallmark for OA (Fig. 7B).

### Discussion

Our findings introduce a new view on TGF-β signaling function with age and OA. Moreover, it provides an explanation for the enigmatic findings in the past and proposes a new mechanism that contributes to understanding OA development.

Growth factors such as TGF-β are able to stimulate chondrocytes in the cartilage of young animals to renew their extracellular matrix (4, 30–35). This has always been viewed as the main function of TGF-β in cartilage. However, during aging and under OA conditions, we have shown that cartilage shows drastically reduced TGF-β signaling via ALK5, as demonstrated by diminished expression of ALK5 and reduced Smad2 phosphorylation (21). In aging mice that do not show cartilage degradation yet, there is already a decrease in the expression of the ALK5 receptor and in Smad2 phosphorylation in cartilage (20). These data indicate that decreased TGF-β signaling through the canonical ALK5 pathway could be a prerequisite to OA development. In endothelial cells, Goumans et al. suggested that the balance between ALK5 and ALK1 determines the outcome of the response to TGF-β (19). Given the reduced ALK5 expression during OA, this led to the hypothesis that a skewed balance between ALK1 and ALK5 during OA favored the ALK1 side leading to an OA phenotype.

From the studies in endothelial cells it has become clear that TGF-β has the ability to signal via ALK1 besides ALK5, thereby phosphorylating Smad1/5/8 (19, 36). Our data show that chondrocytes, like endothelial cells, also express both ALK5 and ALK1 and that they have the ability to respond to TGF-β using both
We show that with age and in OA cartilage, the reduction in ALK5 is far more drastic than the mild reduction in ALK1-positive cells, thus leading toward a skewed balance when compared with normal cartilage. The change in the ratio of Id-1/PAI-1 expression was in line with a shift from a dominant ALK5 to a more pronounced ALK1 signaling. The initial PAI-1 signaling was relatively high, similar to our previous findings of Smad2P expression in young mice (20). Thus, with age, not only is the anabolic function of TGF-β signaling reduced, but the terminal-differentiation potentiating function that we found in vitro becomes more dominant, thereby potentially making aged cartilage prone to develop OA.

ALK1 stimulates the Smad1/5/8 route, which is known to stimulate terminal differentiation and MMP-13 expression in growth plate chondrocytes. It has been frequently hypothesized that OA chondrocytes undergo differentiation toward a hypertrophic-like state. Chondrocytes from OA cartilage, even in unaffected areas, already express markers of hypertrophy when compared with normal cartilage (1, 2, 37). In human OA cartilage, ALK5 correlated with high aggrecan expression as well as high levels of collagen type II expression, both crucial constituents of the cartilage matrix. In contrast, samples with a high ALK1 expression also had a high MMP-13 expression, which is a hallmark of chondrocyte hypertrophy and OA.

As in endothelial cells, also altered signaling in chondrocytes leads to a change in outcome: rather than an anabolic response in a predominant ALK5 signaling situation, a shift in receptor balance favoring ALK1 signaling, either by overexpression of ALK1 or by blocking ALK5, led to elevation of MMP-13. This not only confirms our human cartilage data, but moreover adds a function to our in vitro experiments favoring ALK1 signaling. The initial PAI-1 signaling was in line with a shift from a dominant ALK5 to a more pronounced ALK1 signaling. The initial PAI-1 signaling was relatively high, similar to our previous findings of Smad2P expression in young mice (20). Thus, with age, not only is the anabolic function of TGF-β signaling reduced, but the terminal-differentiation potentiating function that we found in vitro becomes more dominant, thereby potentially making aged cartilage prone to develop OA.

ALK1 stimulates the Smad1/5/8 route, which is known to stimulate terminal differentiation and MMP-13 expression in growth plate chondrocytes. It has been frequently hypothesized that OA chondrocytes undergo differentiation toward a hypertrophic-like state. Chondrocytes from OA cartilage, even in unaffected areas, already express markers of hypertrophy when compared with normal cartilage (1, 2, 37). In human OA cartilage, ALK5 correlated with high aggrecan expression as well as high levels of collagen type II expression, both crucial constituents of the cartilage matrix. In contrast, samples with a high ALK1 expression also had a high MMP-13 expression, which is a hallmark of chondrocyte hypertrophy and OA.

As in endothelial cells, also altered signaling in chondrocytes leads to a change in outcome: rather than an anabolic response in a predominant ALK5 signaling situation, a shift in receptor balance favoring ALK1 signaling, either by overexpression of ALK1 or by blocking ALK5, led to elevation of MMP-13. This not only confirms our human cartilage data, but moreover adds a function to the correlation that was found: changing the receptor balance between ALK1 and ALK5 favoring the ALK1 side has a functional consequence and is able to induce an OA-like phenotype.

Therefore, we postulate that in human OA cartilage, ALK1 signaling apparently stimulates type II collagen degradation via MMP-13 while ALK5 promotes anabolic pathways in chondrocytes, thereby stimulating the synthesis of cartilage matrix molecules. Thus, with age and OA, ALK1 pushes the chondrocytes toward a hypertrophic-like state, expressing MMP-13.

We propose the following hypothesis for OA development and progression. During aging the ALK1/ALK5 ratio increases and Smad1/5/8 signaling (ALK1) becomes dominant relative to Smad2/3 signaling (ALK5). This results in stimulation of articular chondrocytes to differentiate to a chondrocyte with a hypertrophic-like phenotype, expressing high levels of MMP-13. High MMP-13 expression results in degradation of the cartilage collagen network, making the cartilage matrix vulnerable to normal loading. This is the developmental stage of cartilage degeneration in age-related primary OA. When cartilage lesions have developed, a subpopulation of chondrocytes will show high MMP-13 expression, but another subpopulation of chondrocytes in the affected cartilage will show an attempted repair reaction. As a consequence, OA cartilage will be a mixture of cells with a hypertrophic-like phenotype expressing high ALK1 and MMP-13 levels and a population of chondrocytes with high ALK5 expression and high expression of matrix molecules such as aggrecan and type II collagen. The ratio of both populations will be dissimilar in different samples of OA cartilage as a result of OA stage and location of cartilage sampling (distance to severe OA lesions).

A central role for TGF-β in OA becomes apparent. Initially TGF-β signaling via ALK5 in chondrocytes, is a protective factor for articular cartilage, blocking terminal differentiation and MMP-13 synthesis and stimulating the synthesis of matrix molecules. During aging the role of TGF-β changes. Due to variation in receptor expression (ratio ALK1/ALK5), TGF-β becomes a factor stimulating terminal differentiation and MMP-13 expression via ALK1 signaling. A role for TGF-β in OA joints is confirmed by the role of TGF-β in the formation of osteophytes and synovial fibrosis, as we have demonstrated in previous studies (31, 32, 35, 38). TGF-β is protective for cartilage at a young age but is highly involved in OA pathology, inducing cartilage degradation, osteophyte formation, and synovial fibrosis in older individuals. This concept explains our enigmatic findings in earlier studies with regard to TGF-β, with TGF-β being both a potent protective factor for cartilage and a factor that has the ability to induce OA under certain conditions (10–14). Whether TGF-β induction of osteophytes and synovial fibrosis requires ALK5 or ALK1 as a preferred route remains to be investigated. However, a role for ALK1 in fibrosis has been suggested (39). Osteophytes undergo chondrogenic differentiation similar to what is observed in the growth plate. It can be anticipated that the cells forming the osteophytes

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** ALK1 is significantly correlated with MMP-13 expression, whereas ALK5 is significantly correlated with cartilage matrix components in human cartilage. Human cartilage was obtained from patients undergoing knee or hip replacement surgery. RNA was isolated from the cartilage and a quantitative PCR was performed for ALK1, ALK5, aggrecan, collagen type II, and MMP-13. ALK1 expression is significantly correlated with MMP-13 expression (A and D) while ALK5 expression is significantly related to type II collagen and aggrecan expression (A–C). *, p < 0.05; **, p < 0.005; ***, p < 0.001.
require ALK1 signaling at a certain stage. This would make blocking ALK1 during OA even more appealing.

In conclusion, we show that there are changes in ALK1 and ALK5 expression with age and OA favoring dominant ALK1 signaling. Shifting the receptor balance between ALK1 and ALK5 in chondrocytes alters the eventual outcome downstream; in case of aging and OA, this will lead to increased MMP-13 expression. This model provides both an explanation for the development of OA during aging, the shift in chondrocyte phenotype and MMP-13 expression during OA, and provides an explanation for contradictory findings in TGF-β function in cartilage in the past. Our findings not only have implications for the view on OA development, but they also change the way that TGF-β might be used as a repressive factor for cartilage during OA, as the receptor balance within the tissue is crucial for the subsequent downstream response.

Acknowledgments
We thank the Department of Orthopedics, Radbud University Nijmegen Medical Centre Nijmegen, for the collection of human tissue samples.

Disclosures
The authors have no financial conflicts of interest.

References


30. Blaney Davidson, E. N., E. L. Vitters, W. B. van den Berg, and P. M. van der Kraan. 2006. TGF-β-induced cartilage repair is maintained but fibrosis is blocked in the presence of Smad7. Arthritis Res. Ther. 8: R65.


Corrections


Several of the primers listed in Table I were published incorrectly. The results and conclusions in the manuscript are not affected. The corrected Table I is shown below.

Table I. Primers used for quantitative PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>$R^2$</th>
<th>$E'$</th>
<th>Forward Primer (5'→3')</th>
<th>Reverse Primer (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>0.997</td>
<td>2.05</td>
<td>GGCAAATTCAACGGCACA</td>
<td>GTTAGTGGGIGTCTCGTCCTG</td>
</tr>
<tr>
<td>ALK1</td>
<td>0.999</td>
<td>1.90</td>
<td>AAGCTTCTCAAGCTTGGTGA</td>
<td>GCAGAAATTGCTCTCTTGAGT</td>
</tr>
<tr>
<td>ALK5</td>
<td>0.999</td>
<td>2.02</td>
<td>CATCAGGGTGAGATAGTGGT</td>
<td>GTAACACAAAGTGGCCTGGAAT</td>
</tr>
<tr>
<td>Collagen II</td>
<td>0.992</td>
<td>2.15</td>
<td>TTTCCTTACGTCATGAGCA</td>
<td>GACCTTAGGGGAGTTGGAG</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>0.992</td>
<td>2.15</td>
<td>TCTACCCCAACAAAAACGG</td>
<td>AGCGATGCTGCTTGAGCAGT</td>
</tr>
<tr>
<td>MMP13</td>
<td>0.992</td>
<td>1.93</td>
<td>AGACCTTGTTTGCAGACACT</td>
<td>CTTCAGGATTCGCCAGAAGT</td>
</tr>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>0.999</td>
<td>1.92</td>
<td>ATCTTCTTTGCTCCGAGCA</td>
<td>TTCCCCATGGTGTCTGAGC</td>
</tr>
<tr>
<td>ALK1</td>
<td>0.997</td>
<td>2.05</td>
<td>CCATCTGGAATGCGACTG</td>
<td>GGCTGGTGGCAGACATC</td>
</tr>
<tr>
<td>ALK5</td>
<td>0.993</td>
<td>2.05</td>
<td>CGACGCGCTAACAGTGTCTCTTT</td>
<td>CCACCTCACTACAGAATGG</td>
</tr>
<tr>
<td>Collagen II</td>
<td>0.995</td>
<td>2.00</td>
<td>CAGTACACTGCCCCTGAAAGA</td>
<td>CGTAACACAGTCTCCACCAC</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>0.997</td>
<td>2.01</td>
<td>GCCGGCGCTCCTCATGACTG</td>
<td>ATGGACACAGTGCTCTCAC</td>
</tr>
<tr>
<td>MMP13</td>
<td>0.998</td>
<td>1.95</td>
<td>ATTAAGGAGCATGCCGACTTCTT</td>
<td>CCCAGGAGGAAAGACATGAG</td>
</tr>
</tbody>
</table>

Italic text indicates changes made in comparison to the original manuscript.

*aEfficiency.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1090068