EXTENDED REPORT

Loss of transforming growth factor counteraction on interleukin 1 mediated effects in cartilage of old mice

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Objective: To investigate if a difference exists between young and old mice in the response of articular cartilage to interleukin 1 (IL1) and transforming growth factor β (TGFβ) alone or in combination.
Methods: The interaction of IL1 and TGFβ was studied in cartilage of young (three months) and old mice (18 months) both in vivo and in vitro. Therefore, IL1, TGFβ, or IL1 together with TGFβ was injected into the knee joints of mice on days 1, 3, and 5 before harvest of the patellae on day 6. Alternatively, isolated patellae were stimulated with IL1, TGFβ, or IL1 together with TGFβ in culture for 48 hours. Proteoglycan (PG) synthesis and nitric oxide (NO) production were measured.
Results: IL1 inhibited PG synthesis and increased NO production in cartilage of both young and old mice. On the other hand, TGFβ stimulated PG synthesis and reduced NO production in both age groups. Importantly, TGFβ was able to counteract IL1 mediated effects on PG synthesis and NO production in young but not in old mice.
Conclusions: Contrary to the findings in young mice, the cartilage of old animals does not antagonise IL1 effects via TGFβ. This loss of responsiveness to the pivotal cytokine TGFβ on effects of IL1 can be important in the initiation and progression of osteoarthritis (OA).

Osteoarthritis (OA) is a joint disease with a high prevalence in the adult population.[1 2] Although the cause of OA is still unknown, increased age is a major risk factor, with up to 68% of women and 58% of men aged 65 years or older having radiological evidence of OA.[3] Irrespective of the initiating event, OA is caused by an imbalance between anabolic and catabolic processes, which results in a slow but progressive destruction of articular cartilage.

Interleukin 1 (IL1) is considered to be one of the most important catabolic factors in joint diseases.[4–6] In OA large quantities of IL1 are produced by chondrocytes, leading to the production of cartilage degrading matrix metalloproteinases (MMPs).[7–10] Earlier studies have established that IL1 can inhibit cartilage proteoglycan (PG) synthesis and increase cartilage matrix breakdown.[11–13] In old mice, IL1 has been shown to have a prolonged effect on articular cartilage compared with young mice.[14] An important second mediator stimulated by IL1 is nitric oxide (NO),[15] which is also a very potent inhibitor of cartilage PG synthesis.[16] Cartilage from patients with OA produces significant amounts of NO.[17]

A crucial anabolic factor acting on chondrocytes is transforming growth factor β (TGFβ). This is stored in large amounts in articular cartilage and significant levels of active TGFβ are found in the synovial fluid of patients with OA.[7 18 19] TGFβ is thought to play an important part in articular cartilage homoeostasis and during OA.[20 21] We have previously shown that TGFβ can enhance proteoglycan synthesis in vivo in both young and old cartilage.[13 22 23] Furthermore, cartilage degradation mediated by MMPs can be inhibited by TGFβ via up regulation of tissue inhibitors of MMPs (TIMPs 1 and 3).[24 25] Recent studies show that abrogation of the TGFβ signalling pathway in transgenic mice results in osteoarthritic changes of the articular cartilage, further implying a protective role for TGFβ on articular cartilage.[26 27] An important finding is that exogenous addition of TGFβ in knee joints of young mice can counteract cartilage PG depletion and breakdown induced by IL1.[13 23 28] Moreover, PG synthesis was increased by TGFβ in the presence of IL1.

Because OA is a slowly progressive disease, a subtle imbalance between matrix breakdown (IL1, NO) and matrix repair (TGFβ) may be sufficient to initiate the osteoarthritic process. In this study, we compared IL1 and TGFβ responses between the cartilage of young and old mice.

MATERIALS AND METHODS

Animals

Male C57Bl/6 mice aged between three and 21 months were used. Mice were kept in filter top cages with a wood chip bedding under standard pathogen free conditions. They were fed a standard diet and tap water ad libitum. The local animal committee approved this study.

In vivo PG synthesis

Right knee joints of mice aged three months or 18 months were injected with IL1α (1 ng), $TGF\beta_1$ (0.1 μg, R and D systems Europe Ltd, Abingdon, UK) or the combination, in 6 μl PBS supplemented with 0.1% bovine serum albumin. Injections were given three times on alternate days (days 1, 3, and 5). The left knee joint was injected with PBS + 0.1% bovine serum albumin and served as an internal control.

Synthesis of PG was measured ex vivo, one day after the last injection. Mice were killed and the patella with a standard amount of surrounding tissue was dissected as described earlier.[29] Patellae were then pulse labelled with ^{35}S-sulphate (20 μCi, two hours, 37°C), which has previously been shown to be a useful tool to specifically quantify chondrocyte PG synthesis.[29 30] This was followed by washing the patellae extensively with physiological saline and fixing in 96% ethanol for 24 hours at room temperature. Patellae were decalcified in 5% formic acid for four hours at room temperature. Next, articular cartilage was stripped from the underlying bone and dissolved in Lumasolve at 60°C (Lumac, Groningen, The Netherlands). For every patella the incorporation of

Abbreviations: MMPs, metalloproteinases; NO, nitric oxide; OA, osteoarthritis; PG, proteoglycan; IL1, interleukin 1; TGFβ, transforming growth factor β; TIMPs, tissue inhibitors of MMPs

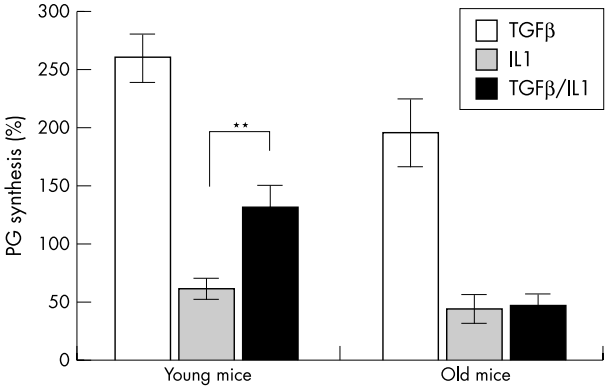

Figure 1 Synthesis of PG in patellar cartilage in old and young mice. Right knee joints of young mice (three months) and old mice (18 months) were injected on days 1,3, and 5 with TGFβ (0.1 μg), IL1α (1 ng), or IL1 + TGFβ. Left knee joints were injected with vehicle and served as internal controls (=100%). On day 6, patellae were isolated and PG synthesis was measured ex vivo by ^{35}S-sulphate incorporation (two hours, 37°C). Each value represents the mean PG synthesis of five patellae. Results were statistically analysed by ANOVA; ** p<0.001.

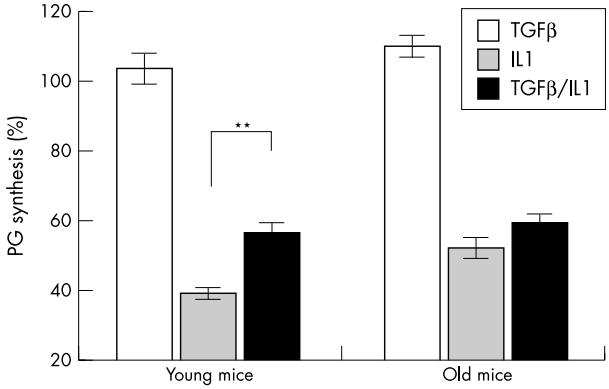

Figure 2 In vitro PG synthesis in patellar cartilage of young (three to five months) and old mice (12–21 months). Patellae were isolated and cultured in RPMI 1640 medium. Patellae were treated for 48 hours with IL1α (10 ng/ml), TGFβ$_1$ (10 ng/ml), a combination of IL1 + TGFβ$_1$, or were not treated. The tissue culture medium was changed every 24 hours. Synthesis of PG was measured via ^{35}S-sulphate incorporation (two hours, 37°C). Incorporation levels of ^{35}S were transformed to percentage compared with control treatment (=100%). Results were statistically analysed by ANOVA; **p<0.001.

^{35}S was measured separately using a liquid scintillation counter, which has been shown to be a reliable method for the determination of PG synthesis in patellar cartilage.[30]

In vitro PG synthesis

Mice were killed between the ages of three and five months or 12 and 21 months. The patella with surrounding tissue was dissected in standardised fashion and placed in RPMI 1640 medium (Dutch modification, Flow laboratories, Irvine, UK). Patellae were divided into four treatment groups (minimum six patellae in each group): no treatment, IL1α (10 ng/ml), TGFβ$_1$ (10 ng/ml), or a combination of IL1 and TGFβ$_1$. The tissue culture medium was changed every 24 hours. After 48 hours of treatment, patellae were labelled for two hours with 20 μCi radioactive sulphate in RPMI 1640 (Dutch modification) supplemented with gentamicine (50 mg/l), 2 mM L-glutamine at 37°C, and 5% CO_2. The rest of the labelling procedure was carried out as described above.

In total 30 "old" patellae and 20 "young" patellae were used in three independent experiments. All absorbances and ^{35}S incorporation levels were transformed to percentages compared with control treatment (=100%). Results were statistically analysed by analysis of variance (ANOVA).

Measurement of NO concentrations

Nitric oxide levels in the culture media were measured on days 1 and 2. The medium concentration of NO_2^- (a stable breakdown product of NO) was determined by Griess reagent using $NaNO_2$ standards. Griess reagent: 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (Sigma Chemicals Co, St. Louis, MO, USA), 1:1 diluted with 1.0% sulphanilamide (*p*-aminobenzene sulphonamide; Sigma Chemical Co, St. Louis, MO, USA) in 5% H_3PO_4. Briefly, 100 μl conditioned medium was mixed with 100 μl Griess reagent in a flat bottom microtitre plate and adsorbance read at 545 nm using an ELISA plate reader (Titertek Multiscan MCC/340).

RESULTS

In vivo PG synthesis

We investigated whether increased age influences the response of articular cartilage to anabolic and catabolic factors. Therefore, IL1, TGFβ$_1$, or IL1/TGFβ were intra-articularly injected on days 1, 3, and 5 in young (three months) and old (18 months) mice. One day later, the effect of these factors on the PG synthesis in isolated patellae was determined.

The dose of TGFβ$_1$ used (100 ng) has been shown to induce maximal effects on PG synthesis in vivo as described earlier.[13–15] The dose for IL1 was based on earlier in vivo experiments.[14 15] In these in vivo studies, the used IL1 concentrations yielded maximal effects on inhibition of PG synthesis. We found no influence of TGFβ or IL1 on the contralateral joint (results not shown).

As figure 1 shows, IL1 induced a more severe suppression of PG synthesis in old than in young animals (56% *v* 39%, respectively) (p<0.05). TGFβ increased PG synthesis 2.5-fold and two-fold in young and old animals respectively. Importantly, TGFβ in combination with IL1 reversed the inhibitory effects of IL1 on PG synthesis to slighly higher than control levels in young but not in old mice. So our results show that TGFβ counteracts IL1 induced suppression of PG synthesis in young mice only.

In vitro PG synthesis

To gain more insight into the mechanisms behind the different responses of old and young cartilage to IL1 and TGFβ, we turned to the in vitro situation. Isolated patellae from young and old mice were cultured for 48 hours with IL1, TGFβ$_1$, or IL1/TGFβ and cartilage PG synthesis was studied.

The dose of TGFβ$_1$ used for in vitro experiments has been shown to induce an optimum effect in cultured chondrocytes.[13] Moreover, this dose seems physiologically relevant as comparable concentrations of TGFβ have been found in arthritic and osteoarthritic joints.[19 31] The IL1 dosage used induced maximal effects in vitro as described in earlier studies.[13 15]

In both age groups, TGFβ only showed a slight increase in PG synthesis compared with controls, a difference that was not significant (fig 2). This loss of TGFβ response is probably due to the short exposure of the cartilage to TGFβ in vitro compared with the repeated exposure to high TGFβ concentrations via bolus injections in vivo. Despite the lack of response after TGFβ addition alone, young mice showed a relative increase of 44% in PG synthesis in the IL1/TGFβ combination group compared with the IL1 group (fig 2, p<0.001). By contrast, old mice did not show a significant increase in PG synthesis in the IL1/TGFβ group compared with IL1 alone. These results showed that TGFβ was able to significantly reduce the IL1 induced inhibition on PG synthesis in young mice, but that in old mice TGFβ could not counteract the IL1 induced suppression of PG synthesis.

Table 1 Influence of IL1 and combination IL1/TGFβ on in vitro NO production (mean (SD) μM) by patellar cartilage of young and old mice

		Young mice	Old mice
Day 1†	Spontaneous	7.81 (0.293)	5.30 (0.175)
	IL1	6.53 (0.463)	8.32 (0.496)
	TGFβ/IL1	5.12 (0.556)*	7.82 (0.436)
	Δ	−28%	−6%
Day 2†	Spontaneous	12.71 (0.390)	10.82 (0.351)
	IL1	18.10 (0.837)	20.82 (0.635)
	TGFβ/IL1	15.0 (0.814)*	20.13 (0.699)
	Δ	−21%	−4%

*Significantly different from IL1 alone; †significantly different from old mice.
Δ=Difference in percentage between IL1 treatment alone and TGFβ/IL1 combination treatment.

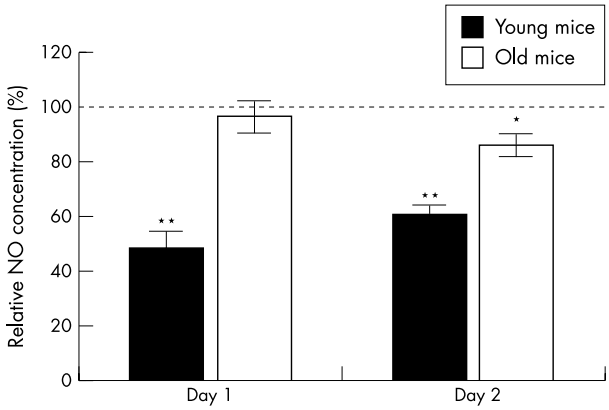

Figure 3 In vitro NO production after TGFβ treatment by patellar cartilage of young (three to five months) and old mice (12 to 21 months). Patellae were isolated, placed in RPMI 1640 medium, and treated for 48 hours with TGFβ (10 ng/ml) or were not treated. The tissue culture medium was changed every 24 hours. NO_2^- in the medium was measured after 24 and 48 hours by the Griess reaction. Production of NO by control treated patellae was stated as 100%. Results were statistically analysed by ANOVA; *p<0.01; ** p<0.001, significantly different from control treatment.

NO synthesis

Nitric oxide is the most important second mediator of IL1 in the inhibition of PG synthesis. TGFβ is known to decrease NO synthesis. Therefore, the NO production was measured in media from control patellae and in patellae after stimulation with IL1, TGFβ, and IL1/TGFβ. Patellae of young and old mice produced NO spontaneously (table 1).

In young mice TGFβ treatment alone resulted in significantly less NO production compared with no treatment, 50% on day 1 and 40% on day 2 (fig 3). In old mice a small but significant effect of TGFβ was found on day 2 (14.4%, $p<0.05$), whereas no effect of TGFβ alone was seen on day 1. Young mice displayed a considerably stronger effect of TGFβ on the inhibition of NO production than old mice, 48% less NO on day 1 and 26% on day 2.

In patellae of young mice, the combination treatment of IL1/TGFβ showed a counteractive effect of TGFβ on NO production induced by IL1 (table 1). TGFβ inhibited IL1 induced NO production by 28% on day 1 ($p<0.05$) and 21% on day 2 ($p<0.01$). In old mice, TGFβ had no significant effect on IL1 induced NO synthesis.

DISCUSSION

Osteoarthritis is a joint disease, which has age as its main risk factor. The determinant that initiates the process is still unknown but the general conception is that an inbalance between catabolic factors and anabolic factors targeting the articular cartilage plays a major part. Because the osteoarthritic process is slowly progressive, it is plausible that a subtle shift in the anabolic/catabolic balance is sufficient to start the process. The aim of this study was to identify differences between young and old cartilage in the influence of anabolic and catabolic factors.

We have previously found that TGFβ increased PG synthesis and content in cartilage when injected intra-articularly in both young and old mice.[13] Moreover, TGFβ supplementation in young mice has been shown to antagonise IL1 mediated inhibition of PG synthesis.[23] However, these studies did not provide any mechanistic information about how TGFβ counteracts the effects of IL1. The underlying in vivo and in vitro study shows that in young mice TGFβ can antagonise IL1 mediated effects on PG synthesis. By contrast, old animals have lost the ability to counteract the effects of IL1 on PG synthesis via TGFβ. We set out to further investigate how increased age influences the response to these cytokines. We hypothesised that the mechanisms behind the counteraction is NO mediated, as NO is the most important second mediator of IL1 and TGFβ is known to decrease NO synthesis.[15 32 33] In addition, we have previously shown that intra-articular injections of IL1 in NO deficient mice did not affect PG synthesis, proving that NO is necessary in mediating IL1 effects.[16] Here we compared NO production of young and old mice after IL1, TGFβ, and IL1+TGFβ stimulation. The results from the NO data showed that old mice had lost the ability to block IL1 induced NO production via TGFβ, whereas young animals had this ability. This was probably not due to loss of the TGFβ response because old animals still showed a strong TGFβ effect on PG synthesis in vivo. However, old mice showed a very strong induction of NO production after IL1 stimulation compared with young animals. The loss of IL1 counteraction by one of the most potent anabolic factors on articular cartilage, together with a hypersensitivity to IL1, could predispose elderly people to OA and contribute to the progression of the disease.

The manner in which TGFβ antagonises IL1 mediated PG synthesis is still unknown but can be based on various mechanisms. TGFβ could cause down regulation of IL1 receptors or increase IL receptor antagonist, the natural inhibitor of IL1. However, as both cytokines are added simultaneously, the TGFβ induced synthesis of IL-Ra will lag considerably behind the direct effect of IL1. On the other hand, IL1 could disturb the TGFβ signalling pathway in old mice, resulting in the loss of TGFβ mediated inhibition of NO synthesis. Consequently, old mice will also be insensitive to endogenous TGFβ, which otherwise would partly inhibit the IL1 effect, as is the case in young animals. Loss of the response to endogenously produced TGFβ could explain the high NO production after IL1 stimulation, as seen in old but not in young mice. Abrogation of the TGFβ pathway by IL1 could potentially be based on upregulation of inhibitory molecules such as the recently described Smad-6 and Smad-7.[34–36] In addition, in old mice IL1

might block essential secondary mediators in the TGFβ signalling pathway such as receptor associated Smad-2, Smad-3, or the common mediator Smad-4.[34]

The results show a discrepancy between the in vivo and in vitro situation in the PG synthesis response to TGFβ. Whereas in vivo TGFβ greatly enhances PG synthesis in both age groups, no effect of TGFβ addition was found in the in vitro situation. This difference is probably caused by the mode of TGFβ administration: multiple high bolus concentrations in vivo versus a continuous relatively low dose in vitro. As we have previously shown, TGFβ has a slow response time in vivo; therefore, 48 hours might be too early for the direct TGFβ response on PG synthesis in the in vitro situation.

Our results indicate that in cartilage of young but not old animals, TGFβ is able to counteract the deleterious effects of IL1 on PG synthesis and NO production. By contrast, chondrocytes from old animals seem to have lost the ability to antagonise IL1 effects via TGFβ. Taken together, in old animals the balance between anabolic and catabolic factors seems to have shifted towards the catabolic side, a phenomenon that might be related to the initiation and progression of OA.

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