EUROPEAN JOURNAL OF CANCER 46 (2010) 616–624

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

COX-2 expression in chondrosarcoma: A role for celecoxib treatment?

Y.M. Schrage [a], I. Machado [b], D. Meijer [a], I. Briaire-de Bruijn [a], B.E. van den Akker [a], A.H.M. Taminiau [c], T. Kalinski [d], A. Llombart-Bosch [b], J.V.M.G. Bovée [a,*]

[a] Department of Pathology, Leiden University Medical Center, Leiden, The Netherlands
[b] Department of Pathology, University of Valencia, Valencia, Spain
[c] Department of Orthopedic Surgery, Leiden University Medical Center, Leiden, The Netherlands
[d] Department of Pathology, Otto-von-Guericke-University, Magdeburg, Germany

ARTICLE INFO

Article history:
Received 4 September 2009
Accepted 10 November 2009
Available online 11 December 2009

Keywords:
Bone tumours
Chondrosarcoma
COX-2 inhibition
Therapy
Xenograft

ABSTRACT

Chondrosarcomas are resistant to conventional chemo- and radiotherapy. A subset of chondrosarcomas arises secondarily in the benign tumour syndromes enchondromatosis (EC) and multiple osteochondromas (MO), and prevention of tumour development would greatly improve prognosis. We therefore investigated the effect of selective COX-2 inhibition on chondrosarcoma growth.

COX-2 expression was studied in central- and peripheral cartilaginous tumours. The effect of COX-2 inhibition was assessed in four high-grade chondrosarcoma cell lines using celecoxib and NS-398 treatment. COX-2 activity (prostaglandin E_2 (PGE_2) ELISA) and cell viability were measured. The (prophylactic) effect of celecoxib on chondrosarcoma growth *in vivo* was studied for 8 weeks using a xenograft model of cell line CH2879 in immunoincompetent nude mice.

High COX-2 protein expression was mainly found in solitary peripheral chondrosarcoma and in enchondromatosis-related central chondrosarcoma, which was confirmed by qPCR. After 72 h of celecoxib treatment, a significant decrease in cell viability was observed in three chondrosarcoma cell lines. *In vivo*, celecoxib initially slowed tumour growth in chondrosarcoma xenografts; however, after prolonged treatment relapsed tumour growth was observed. Tumour volume was negatively associated with celecoxib serum levels, and seemed smaller in the high-dose prophylactic treatment group.

We confirmed the expression of COX-2 in 65% of chondrosarcomas, and COX-2 inhibition by celecoxib diminished cell viability *in vitro*. The initial response and the decrease in tumour volume with increased celecoxib serum levels *in vivo* supported a role for celecoxib, although relapsed tumour growth after 6 weeks was worrisome. Also the role of high-dose prophylactic celecoxib in preventing the development of benign and malignant cartilage tumours in EC and MO patients deserves further investigation.

* *Corresponding author:* Address: Department of Pathology, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands. Tel.: +31 715266617; fax: +31 715266952.
E-mail address: J.V.M.G.Bovee@lumc.nl (J.V.M.G. Bovée).
0959-8049/$ - see front matter
doi:10.1016/j.ejca.2009.11.002

1. Introduction

Chondrosarcoma of bone is a malignant cartilage-forming tumour, which is highly insensitive to classical chemotherapeutics and radiation therapy. Chondrosarcomas are histologically divided into three grades, which is currently the only objective predictor of metastasis. Grade I tumours rarely metastasise with a 10-year survival rate of 83%, while a 10-year survival rate for grade III tumours decreases to 29% due to metastatic disease.[1] Marginal or intralesional excision of tumours can result in local recurrence with increased histological grade.[2] Currently, surgical removal of the tumour is the only option for curative treatment. There is no treatment with curative intent for patients with metastatic disease or inoperable tumours.

The majority (80–85%) of conventional chondrosarcomas arise in the medullar cavity of the bone and are designated as primary central chondrosarcomas.[3] For less than 1% of central chondrosarcomas, there is clinical evidence of a pre-existing (benign) enchondroma.[3,4] Enchondromas occur mostly as solitary lesions, although they may occur as multiple lesions in the context of enchondromatosis (Ollier disease), which is a rare non-hereditary syndrome.[5] The risk of malignant progression is increased up to 30–35% in enchondromatosis patients[6] as compared to solitary enchondroma (<1%).

At the surface of the bone, peripheral chondrosarcomas arise secondary to a pre-existing osteochondroma. Multiple osteochondromas (MO) is an autosomal dominant hereditary disorder which occurs in children and young adolescents. Malignant progression of hereditary osteochondroma is slightly more frequent than that of solitary lesions (1–5% versus 1%). Preventing new tumour formation and malignant progression in enchondromatosis and multiple osteochondroma patients would greatly benefit their prognosis.

In colorectal cancer, a protective effect of prostaglandin synthesis inhibitors (also known as non-steroidal anti-inflammatory drugs (NSAIDs)) has been suggested against development and growth of the tumours. Celecoxib and rofecoxib, both selective COX-2 inhibitors, were shown to reduce the number and size of colorectal polyps in the adjuvant treatment of Familial Adenomatous Polyposis (FAP) patients.[7,8] Also aspirin was found to have a chemopreventive effect on adenoma recurrence in patients in whom a non-FAP-related adenoma had been removed.[9] NSAIDs block attachment sites for arachidonic acid on the COX enzyme, thereby inhibiting prostaglandin production.[10] Whereas COX-1 is constitutively expressed under physiologic conditions, COX-2 is induced by cytokines and free radicals, making it a suitable target for (anti-cancer) therapy.

Endo et al. reported high COX-2 expression in a substantial amount of chondrosarcomas (16/72) by immunohistochemistry, which was associated with high histological grade and poor prognosis.[11] However, Sutton found no correlation of COX-2 protein expression and histological grade in 24 chondrosarcomas (6/9 grade I tumours; 4/6 grade II tumours and 1/6 grade III tumours), whereas 8 enchondromas were negative.[12]

We investigated whether COX-2 inhibition could play a role in either the treatment of high-grade chondrosarcomas or the prevention of malignant progression of tumours associated with enchondromatosis or multiple osteochondromas. Therefore, we determined COX-2 mRNA and protein expression in patient material. We investigated the effects of COX-2 inhibition on COX-2 protein expression, PGE_2 levels, and cell viability in four high-grade chondrosarcoma cell lines *in vitro*. In addition, a chondrosarcoma xenograft model of immunoincompetent nude mice was used to study the effects of COX-2 inhibition *in vivo*.

2. Materials and methods

2.1. COX-2 expression in patient material of cartilaginous tumours

Conventional central and peripheral cartilaginous tumours were selected based on accepted clinicopathological and radiological criteria.[3] Juxtacortical-, mesenchymal-, dedifferentiated- and clear-cell chondrosarcomas were excluded. In total, formalin-fixed paraffin-embedded specimens from 66 patients (Table 1) and fresh-frozen material from 34 patients were studied. Histological grading was performed according to Evans.[1] All specimens were handled according to the ethical guidelines described in 'Code for Proper Secondary Use of Human Tissue in The Netherlands' of the Dutch Federation of Medical Scientific Societies.

COX-2 immunohistochemistry (Table 2) of tumour tissue was independently semi-quantitatively scored for cytoplas-

Table 1 – COX-2 protein expression in cartilaginous tumours.

	Central						Peripheral							
	Solitary		EC		Total		Solitary		MO		Total		Overall	
	pos	%	pos	%	pos	%	pos	%	pos	%	pos	%	pos	%
Benign	3/9	33	0/6	0	3/15	20	2/8	25	1/9	11	3/17	18	6/32	19
Malignant	1/6	17	6/6	100	7/12	58	12/13	92	3/9	33	15/22	68	22/34	65
Grade I CS	0/4	0	1/1	100	1/5	20	6/6	100	2/5	40	8/11	73	9/16	56
Grade II CS	1/2	50	3/3	100	4/5	80	6/7	86	1/4	25	7/11	64	11/16	69
Grade III CS	–	–	0/2	0	0/2	0	–	–	–	–	–	–	0/2	0
ALL (benign + malignant)	4/15	27	6/12	50	10/27	37	14/21	67	4/18	22	18/39	46	28/66	42

Table 2 – Antibodies and protocols used for immunohistochemistry and immunoblotting.

Antigen	Clone	Application	Manufacturer	Origin	Against	Positive control	Pre-incubation	Secondary	Antibody concentration	Antigen retrieval
COX-2	PG-46	IHC (human)	Nuclilab	Rabbit	Human	Colorectal carcinoma	60′ blocking solution[b]	Anti-rabbit HRP	1:100	Citrate
COX-2	PG-46	IHC (mouse)	Nuclilab	Rabbit	Human	Colorectal carcinoma	60′ blocking solution[a]	Anti-rabbit envision[a]	1:100	Citrate
Ki-67	MIB-1	IHC (mouse)	Dako	Mouse	Human	Any tumour	None	Anti-mouse IgG1/HRP	1:100	Citrate
Cleaved caspase-3 (Asp175)		IHC (mouse)	Cell signalling	Rabbit	Human	Burkitt lymphoma	None	Anti-rabbit envision	1:100	Citrate
CD31 (Pecam-1)		IHC (mouse)	Santa Cruz	Rabbit	Mouse	Any	30′ NGS 5%	Anti-rabbit envision	1:500	None
COX-2		IB	Cayman chemicals	Mouse	Human	Colorectal carcinoma	60′ 5% non-fat dry milk in PBS/0.05%Tween	Anti-mouse HRP	0.25 ng/ml	
Tubulin		IB		Mouse	Human	Any	60′ 5% non-fat dry milk in PBS/0.05%Tween	Anti-mouse HRP	1:1000	

IHC: Immunohistochemistry, IB: Western blot and NGS: normal goat serum.
a For human tissue anti-rabbit HRP was used.
b Blocking solution: 0,01 M Tris, 0,1 M $MgCl_2$, 5% Tween 20, 1% BSA and 5% normal goat serum.

mic staining, as described previously,[13] without knowledge of the clinicopathological data. Scores were given for intensity (1 = weak, 2 = moderate, 3 = strong) and percentage of positive cells (1 = 0–24%, 2 = 25–49%, 3 = 50–74% and 4 = 75–100%). To avoid tumours with single positive cells being regarded as positive, a cut-off level of total sum $\geqslant 3$ was applied.

RNA was isolated from fresh frozen tumour tissue of 34 central tumours as described previously.[14] Growth plate samples ($n = 4$) were used as normal counterpart controls. Messenger RNA expression of *COX-2* was studied using quantitative RT-PCR (forward primer *GAATCATTCACCAGGCAAATTG*, reverse primer *TCTGTACTGCGGGTGGAACA*), as previously described.[15] For normalisation GENORM was used.[16]

2.2. *Inhibition of COX-2 in chondrosarcoma in vitro*

Chondrosarcoma cell lines derived from chondrosarcoma grade II (SW1353, American Type Culture Collection (ATCC), Manassas, VA), grade III (CH2879[17] and OUMS27[18]) and a recurrent chondrosarcoma grade II in enchondromatosis (C3842[19]) were cultured in RPMI 1640 supplemented with 10% heat-inactivated foetal calf serum (Gibco, Invitrogen Life-Technologies, Scotland, UK). Cell line HT29 (ATCC), expressing high COX-2 levels, was used as a control. Cells were grown at 37 °C in a humidified incubator with 95% air and 5% CO_2. The cartilaginous phenotype of the chondrosarcoma cell lines was confirmed by RT-PCR, showing mRNA expression of *collagens I*, *2B*, *3* and *10*; *Aggrecan*; and *SOX9*.[20]

Protein extraction and immunoblotting were performed as described previously.[21]

A WST-1 colorimetric assay (Roche Diagnostics GmbH, Penzberg, Germany) was used as described previously[21] to measure metabolic activity representing the amount of viable cells in response to celecoxib (Pfizer, NY, USA) and NS-398 (Cayman chemicals, Ann Arbor, MI, USA). 5.0×10^3 chondrosarcoma cells (CH2879, OUMS27 and C3842) and 1.5×10^3 HT29 and SW1353 cells were seeded. Celecoxib and NS-398 were diluted in DMSO. After 24 h, increasing concentrations of the drugs (5, 10 and 25 μM) or 0.1% DMSO were added to the culture medium and treatment was performed for 72 h. CS3842 was not included in the NS-398 assay.

For measuring PGE_2 levels, cells of OUMS27, CH2879, C3842 and HT29 were seeded in a 24-well plate at a density of 5.5×10^4, and SW1353 was seeded at a density of 2.0×10^4. Twenty-four hours after seeding, cells were treated with celecoxib in increasing dosages (5, 10 and 25 μM) or with 0.1% DMSO. After 72 h, PGE_2 concentration in the medium was determined by a PGE_2-specific enzyme-linked immunoassay (Cayman chemicals, Ann Arbor, MI, USA) according to the manufacturer's protocol. mRNA was extracted and CYP19A1 mRNA levels were determined to assess aromatase activity.[20] Five percent serum supplementation was used for all experiments.

2.3. *In vivo chondrosarcoma model*

Sixty Swiss male nude mice (Crl:NU(Ico)-Foxn1nu (IFFA-CREDO, Lyon, France)) were randomly assigned to one of five groups (Fig. 1). Low doses contained 500 ppm celecoxib, high doses contained 1000 ppm. The selective COX-2 inhibitor

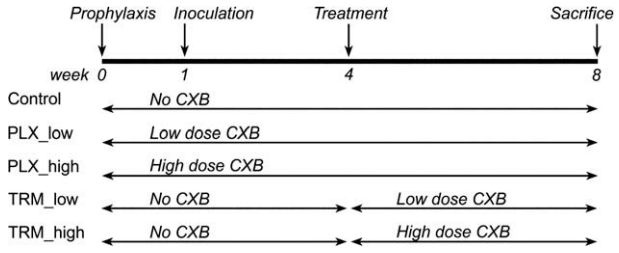

Fig. 1 – Sixty immunoincompetent nude mice were randomly assigned to either prophylactic low-dose celecoxib (PLX_low), prophylactic high-dose celecoxib (PLX_high), treatment low-dose celecoxib (TRM_low), treatment high-dose celecoxib (PLX_high) or control group (Control).

celecoxib (CS-58635) was incorporated into a modified nude mouse diet (Altromin Gesellschaft fuer Tierernaehrung mbH, Lage, Germany) and irradiated to accomplish sterility. Mice were able to feed and drink ad libitum. The amount of food consumed was monitored individually. Prophylaxis was started 7 d prior to inoculation, as steady-state levels of celecoxib are reached in 5 d in humans (Pfizer). At week 1 all mice were injected with 2×10^6 CH2879 cells, subcutaneously on the back. Since chondrosarcomas have a relatively slow growth rate, we waited for 21 d for all tumours to reach a size of at least 4.0×4.0 mm, before treatment with celecoxib was started. Treatment was prolonged for 30 d, after which the mice were sacrificed. The mice were weighted once a week. Calipers were used to measure the tumour volume *in vivo* using the following formula: (length × width^2)/2. Blood samples, obtained by aortal punction at the time of sacrifice, were allowed to coagulate and were centrifuged for 10 min at 13,000 rpm; supernatants were collected and stored at −20 °C until HPLC analysis. HPLC was performed by Case Bel (Borgerhout, Belgium) as described previously.[22]

The excised tumours were fixed in 0.1% formalin for 24 h and embedded in paraffin. To study the possible toxicity of celecoxib, tissue of the heart, lungs, liver and kidney were taken at autopsy for histological analysis. Xenografted tumours were analysed by H&E, Toluidine blue and immunohistochemistry for COX-2, Ki-67, cleaved caspase-3 and CD31 (Table 2). COX-2 staining was scored as negative, very weak or positive. Ki-67 and cleaved caspase-3 staining were digitally scored using confocal microscopy (Nuance 2.6.0 Cambridge Research and Instrumentation Inc., Woburn, MA, USA). A minimum of 2000 cells were counted and the percentage of positive cells was automatically calculated (ImageJ 1.37V, Wayne Rasband, National Institutes of Health, USA). To assess microvessel density, CD31-positive vessels were counted in 10 high-power fields per tumour. An independent experiment was performed using grade II chondrosarcoma xenograft model, with high celecoxib prophylaxis ($n = 12$) and treatment ($n = 12$) group.

2.4. Statistics

Comparison between groups was performed using Pearson χ^2 (immunohistochemistry) and Student's *t*-test (RT-PCR). *P* values < 0.05 were considered significant.

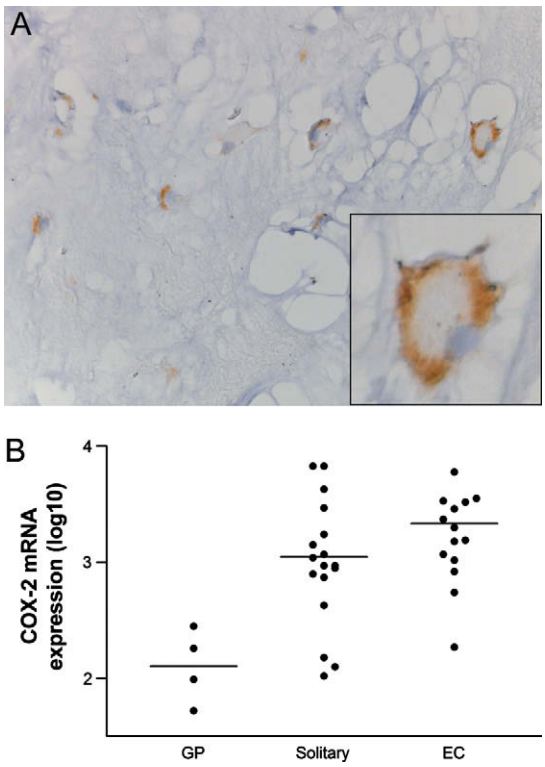

Fig. 2 – (A) Cytoplasmic COX-2 expression in central chondrosarcoma grade I. (B) COX-2 mRNA was higher expressed in central cartilaginous tumours compared to growth plate (GP) samples, and tumours related to enchondromatosis (EC) showed higher expression of COX-2 than solitary tumours.

3. Results

3.1. Higher COX-2 expression in chondrosarcoma and in enchondromatosis

In total, in 65% of the chondrosarcomas tumour cells demonstrated cytoplasmic COX-2 protein expression (Fig. 2A). Malignant tumours were more often positive for COX-2 than benign tumours (58% versus 20%, $p = 0.040$ for central and 68% versus 18%, $p = 0.002$ for peripheral, Pearson χ^2), although numbers were small. In the group of central chondrosarcomas positivity was mainly seen in enchondromatosis-related chondrosarcomas (6/6), whereas peripheral solitary tumours were more often positive (12/13) than MO-related chondrosarcomas (3/9). Results are summarised in Table 1.

Also at mRNA level enchondromatosis-related tumours tend to show higher COX-2 expression, as compared to solitary tumours ($p = 0.056$ Student's *t*-test) (Fig. 2B). However, no difference in COX-2 mRNA expression between benign ($n = 7$) and malignant tumours ($n = 27$) was found (Student's *t*-test $p = 0.58$) (not shown).

3.2. COX-2 inhibitors decrease proliferation of chondrosarcomas in vitro

All four chondrosarcoma cell lines demonstrated COX-2 protein expression in variable levels (Fig. 3A). After 72 h of

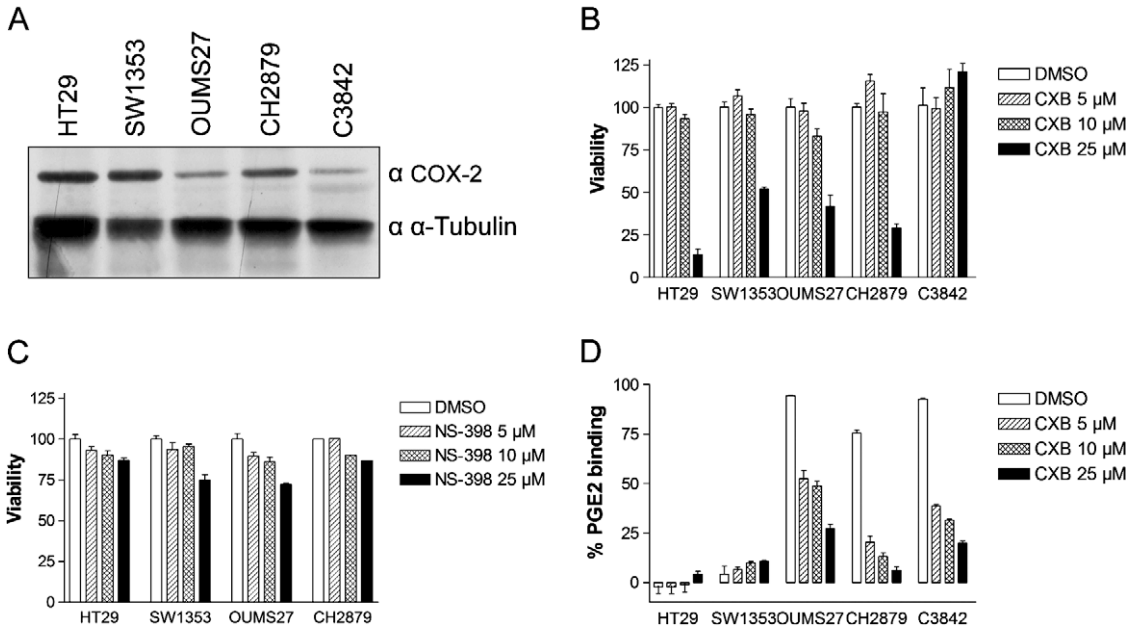

Fig. 3 – (A) COX-2 protein expression in cell lines (B) decreased viability (SW1353, OUMS27 and CH2879) upon 25 μM celecoxib treatment. (C) NS-398 has a moderate effect. (D) COX-2 activity is absent in SW1353 and decreased upon 5 μM celecoxib treatment in OUMS27, CH2879 and C3842.

25 μM celecoxib treatment, a decrease in cell viability was observed, comparable to HT29, in the three cell lines that were derived from solitary tumours (Fig. 3B). For OUMS27 a decrease in cell viability was observed already at 10 μM celecoxib. C3842 did not respond to celecoxib (Fig. 3B), not even when treatment was prolonged to 120 h (data not shown). Effects of NS-398 were more subtle (Fig. 3C).

3.3. *Response to celecoxib is independent of COX-2 activity*

By PGE_2 ELISA we showed that in CH2879, OUMS27 and C3842 the COX enzymes are active and that a dose of 5.0 μM celecoxib was enough to significantly decrease PGE_2 levels (Fig. 3D). Remarkably, we were not able to detect COX activity in HT29 and SW1353, which responded well to celecoxib treatment. Relative CYP19A1 mRNA levels were decreased twofold upon celecoxib treatment in SW1353, whereas in CH2879 and C3842 CYP19A1 levels were increased twofold (data not shown).

3.4. *Celecoxib initially slows tumour growth in chondrosarcoma xenografts*

During the *in vivo* study, all mice had comparable body weights and amounts of food administered (Fig. 4A). Six mice died during the experiments, in both the celecoxib and the control groups. Serum levels of celecoxib corresponded to the dose of celecoxib administered, although the levels were variable (Fig. 4B). Tumour size was negatively correlated to celecoxib serum levels ($r = -0.39$) (Fig. 4C). At 4 weeks, the tumour volume seemed smaller in the groups receiving celecoxib prophylaxis, which was significant for the high dose, as compared to that in the control group (Student's *t*-test $p = 0.053$ (low dose) and $p = 0.028$ (high dose), respectively) (Fig. 4D). Histological evaluation of the heart, lungs, liver, spleen and kidneys of the mice did not reveal any signs of toxicity.

3.5. *Relapse of tumour growth in xenografts after prolonged treatment*

At week 6 a relapse was observed, most clearly in the low celecoxib prophylaxis and treatment groups. At the end of the experiment (week 8), only the high prophylaxis group showed smaller tumour volumes than the control group (Fig. 4E). However due to the large variation in tumour volume within the groups, statistical calculations are not meaningful. The growth curves of the tumours showed that at week 6 the tumours started to grow even faster than those in the control group (Fig. 4F). This was exactly the time point at which the growth curves of the mice flattened (Fig. 4A), suggesting the end of puberty. The independent experiments, in which high celecoxib doses were used, showed similar results for the grade II chondrosarcoma xenograft model (data not shown).

3.6. *Evaluation of tumour tissue*

Tumours were highly cellular with limited cytonuclear atypia and a limited amount of extracellular matrix (Fig. 5A). Toluidine-blue staining confirmed the deposition of proteoglycans (Fig. 5B). Whereas in all celecoxib-treated groups COX-2 staining was absent or very weak (Fig. 5C), strong COX-2 expression was observed in 50% of the control tumours (Fig. 5D). Proliferation was seen in all groups (Fig. 5E), with higher Ki-67 expression in the treated tumours at week 8 (Student's *t*-test low dose $p = 0.040$ and high dose $p = 0.018$) (Asterisks, Fig. 5F). Cleaved caspase-3 expression was low (mean 0.746%, range 0.2–1.7%), as was microvessel density (mean

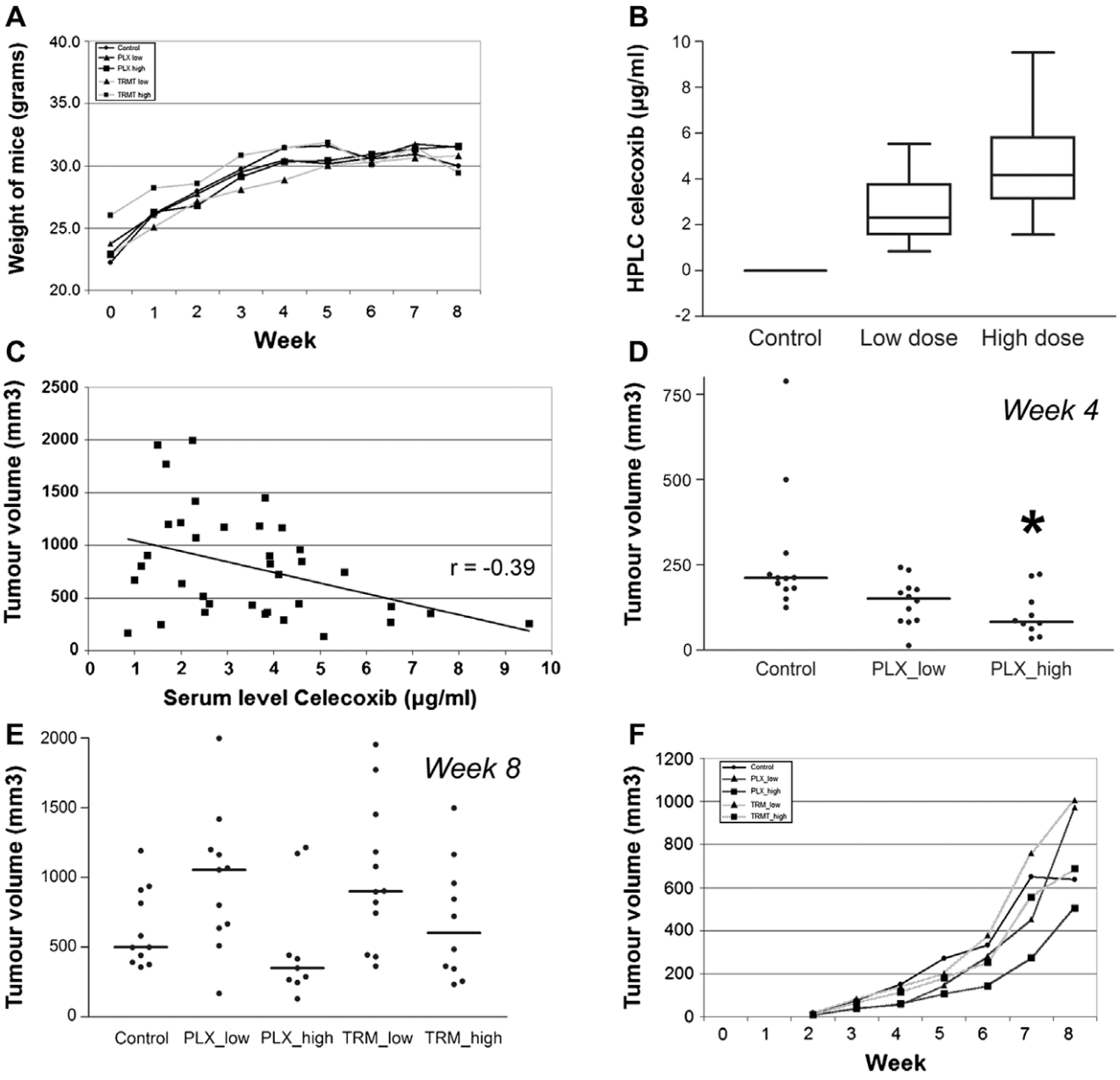

Fig. 4 – (A) Mice body weight increases till week 6. (B) Celecoxib blood levels correspond to celecoxib dose. (C) Tumour volume is negatively correlated with celecoxib serum levels ($r = -0.39$). (D) Lower tumour volume in prophylaxis group at week 4. (E) Tumours exposed to low-dose celecoxib are larger than controls at week 8, although variation within the groups is considerable. (F) Overall growth curve of the tumours showing relapse at week 6 for those exposed to low-dose celecoxib. (*: significantly different from controls).

18.9 per 10 hpf, range 4.0–40.0 per hpf) and no differences between groups were found (Figs. 5G and H).

4. Discussion

Since there is nothing to offer with curative intent to patients with metastatic or inoperable chondrosarcoma, there is a desperate need for new therapeutic options. Furthermore, the prevention of development of new tumours and especially of malignant transformation of benign precursor lesions in patients with enchondromatosis and multiple osteochondromas would greatly improve the prognosis of these children and young adults. In this study, we investigated the potential of selective COX-2 inhibition for the treatment of chondrosarcoma.

We demonstrated the expression of COX-2 in a subset of enchondromas (20%), osteochondromas (18%), and central (58%) and peripheral (68%) chondrosarcomas, comparable to published results.[11,12] Interestingly, COX-2 protein and mRNA expression was mainly found in enchondromatosis-related tumours, whereas correlation with histological grade as found by IHC could not be confirmed on mRNA level.

Chondrosarcoma cell viability decreased after administration of high (super-physiologic) levels of celecoxib and NS-

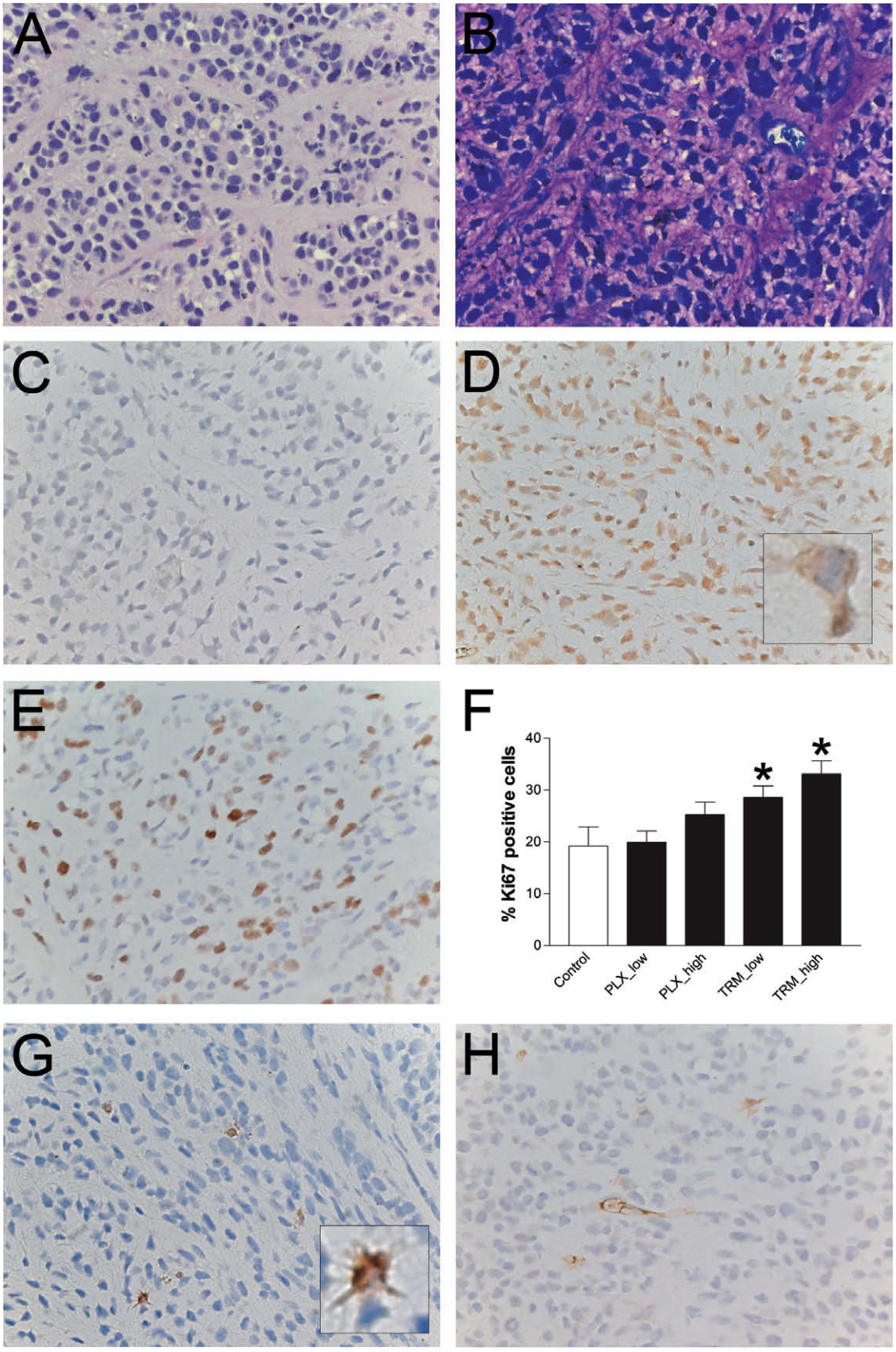

Fig. 5 – (A) H&E staining of the CH2879 xenograft. (B) Toluidine blue staining confirmed proteoglycan content. (C) Absent or very weak COX-2 staining in treated tumours. (D) Strong COX-2 staining in 50% of the controls. (E and F) Higher proliferation rate (Ki-67) in the celecoxib-treated tumours. (G) Caspase 3-mediated apoptosis is limited. (H) Microvessels detected by CD31.

398, while a physiologic dosage of celecoxib was able to abolish most COX-2 activity in three cell lines. Despite the lack of COX-2 activity in SW1353, a decrease in cell viability was found in response to celecoxib, which suggests a COX-2 independent mechanism, which was previously suggested for colorectal cancer (reviewed in [23]). In breast cancer COX-2 inhibitors were found to suppress aromatase activity in both a PGE_2-dependent manner and a PGE_2-independent manner,[24] and Cleton-Jansen et al. showed that chondrosarcoma growth could be inhibited by aromatase inhibitors *in vitro*.[20] The decrease in CYP19A1 activity in SW1353 during celecoxib treatment suggests that this growth inhibitory effect is exerted via the inhibition of aromatase.

Since three of four high-grade chondrosarcoma cell lines responded to celecoxib *in vitro*, we also tested celecoxib in chondrosarcoma xenografts, especially since celecoxib acts through inhibition of angiogenesis of which the effect cannot be evaluated *in vitro*. Although chondrosarcomas in patients are usually large (>5 cm), poorly vascularised unless high grade, with accession of cancer drugs additionally compromised by the sometimes abundant hyaline cartilaginous matrix, the chondrosarcoma xenograft mouse model is at present the best available model to study chondrosarcoma *in vivo*. Celecoxib treatment was initially effective in slowing the growth rate of chondrosarcoma. Moreover, tumour size was inversely correlated with celecoxib serum levels, measured at the end of the experiment. However, a relapse was observed in week 6, which was especially prominent in the mice receiving low-dose celecoxib. In addition, the treated tumours showed an increased proliferation rate. Interestingly, at week 6 the growth curve of the mice flattened, suggesting the end of puberty, which suggests hormonal influences on tumour growth. Here, this effect cannot be attributed to oestrogens, since CH2879 is oestrogen receptor negative.[17]

One of the mechanisms of tumour inhibition of celecoxib is thought to be the inhibition of angiogenesis. The relapse at week 6 might reflect a time point where celecoxib can no longer inhibit angiogenesis allowing vessel ingrowth. At sacrifice no differences in microvessel density were found, suggesting that either differences in microvessel density are completely overcome or a different mechanism was responsible for the initial decreased tumour growth. Unfortunately, we were not able to study tumour characteristics or blood parameters during the experiment.

In analogy to the prevention of new adenoma formation in familial adenomatous polyposis, prevention of development and malignant transformation of enchondromas and osteochondromas in patients with enchondromatosis and multiple osteochondromas might be beneficial. Although we showed COX-2 expression to be higher in enchondromatosis-related tumours than in solitary tumours, the enchondromatosis-derived cell line C3842 did not respond to celecoxib treatment *in vitro*. *In vivo*, a growth-inhibiting effect was shown in the first 4 weeks of the study, in the prepubertal mice, which was abolished at the moment the mice reached adulthood. This might suggest that celecoxib is more effective in prepubertal patients. Moreover, there was a trend for high-dose prophylactic celecoxib treatment to have smaller tumours at the end of the study. However, the high-dose prophylaxis group showed a higher proliferation rate as compared to the control group. Thus, our results are not conclusive on whether the paediatric population of multiple osteochondromas and enchondromatosis patients might benefit from celecoxib treatment and further studies should be performed. In addition, it should be noted that our model is suboptimal to study the effect of celecoxib on the prevention of malignant transformation, since we used high-grade chondrosarcoma xenografts. Unfortunately, there is no suitable *in vivo* model for enchondromatosis or multiple osteochondromas, and it is difficult to obtain xenografts from low-grade chondrosarcomas.

During long-term clinical trials, COX-2 inhibitors were shown to have cardiovascular side-effects. Celecoxib trials were discontinued earlier[25] and rofecoxib was withdrawn by the FDA.[26] However, children and adolescents are at low risk of cardiovascular disease, which renders the use of celecoxib relatively safe. Accordingly, celecoxib is prescribed to juvenile rheumatoid arthritis patients from the age of 2 years (reviewed in [27]). Although toxic effects of celecoxib were not found on histological evaluation of internal organs of the mice in our study, long term safety was not studied here. Next to the potential anti-tumour effect of celecoxib, its analgetic effect will be beneficial to multiple osteochondromas and enchondromatosis patients.

In conclusion, we confirmed the expression of COX-2 in 65% of chondrosarcomas, and COX-2 inhibition diminished cell viability *in vitro*, although this was independent of COX-2 activity. COX-2 inhibition in our grades II and III chondrosarcoma xenograft model also suggested an anti-tumourigenic effect of celecoxib, since tumour size was negatively correlated to celecoxib serum levels. In addition, since at week 4 the tumour volume was significantly smaller in the group receiving high-dose prophylaxis, celecoxib might be beneficial in the prevention of benign and malignant cartilage tumour development in prepubertal EC and MO patients. However, further studies are warranted since worrisome tumour-promoting effects were observed after the 6th week.

Conflicts of interest statement

None declared.

Acknowledgements

The authors like to thank P.J.K. Kuppen (Department of Surgery, LUMC, The Netherlands), P.C.W. Hogendoorn (Department of Pathology, LUMC, The Netherlands), M. Karperien (Department of Tissue Regeneration, University of Twente, The Netherlands) and C.P.E. Asselbergs (Department of Surgery, LUMC, The Netherlands) for fruitful discussion, and F. Prins, A. van der Wal and P.Wijers-Koster for excellent technical assistance.

Grant support: Netherlands Organisation for Scientific Research [908-02-018; Y.M.S., and 917-76-315 to J.V.M.G.B.]. This study was performed within the context of the EuroBoNeT consortium [018814], a European Commission granted Network of Excellence for studying the pathology and genetics of bone tumours.

REFERENCES

1. Evans HL, Ayala AG, Romsdahl MM. Prognostic factors in chondrosarcoma of bone. A clinicopathologic analysis with emphasis on histologic grading. *Cancer* 1977;**40**:818–31.
2. Brien EW, Mirra JM, Luck Jr JV. Benign and malignant cartilage tumors of bone and joint: their anatomic and theoretical basis with an emphasis on radiology, pathology and clinical biology. II. Juxtacortical cartilage tumors.. *Skeletal Radiol* 1999;**28**:1–20.
3. Bertoni F, Bacchini P, Hogendoorn PCW. Chondrosarcoma. In: Fletcher CDM, Unni KK, Mertens F, editors. *World Health Organisation classification of tumours. Pathology and genetics of tumours of soft tissue and bone*. Lyon: IARC Press; 2002. p. 247–51.
4. Lucas DR, Bridge JA. Chondromas: enchondroma, periosteal chondroma, and enchondromatosis. In: Fletcher CDM, Unni KK, Mertens F, editors. *World Health Organization classification of tumours. Pathology and genetics of tumours of soft tissue and bone*. Lyon: IARC Press; 2002. p. 237–40.
5. Ollier M. Dyschondroplasie. *Lyon Med* 1900;**93**:23–5.
6. Schwartz HS, Zimmerman NB, Simon MA, Wroble RR, Millar EA, Bonfiglio M. The malignant potential of enchondromatosis. *J Bone Joint Surg Am* 1987;**69**:269–74.
7. Steinbach G, Lynch PM, Phillips RK, et al. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *New Engl J Med* 2000;**342**:1946–52.
8. van Stolk R, Stoner G, Hayton WL, et al. Phase I trial of exisulind (sulindac sulfone, FGN-1) as a chemopreventive agent in patients with familial adenomatous polyposis. *Clin Cancer Res* 2000;**6**:78–89.
9. Baron JA, Cole BF, Sandler RS, et al. A randomized trial of aspirin to prevent colorectal adenomas. *New Engl J Med* 2003;**348**:891–9.
10. Smith WL, Marnett LJ, DeWitt DL. Prostaglandin and thromboxane biosynthesis. *Pharmacol Ther* 1991;**49**:153–79.
11. Endo M, Matsumura T, Yamaguchi T, et al. Cyclooxygenase-2 overexpression associated with a poor prognosis in chondrosarcomas. *Hum Pathol* 2006;**37**:471–6.
12. Sutton KM, Wright M, Fondren G, Towle CA, Mankin HJ. Cyclooxygenase-2 expression in chondrosarcoma. *Oncology* 2004;**66**:275–80.
13. Bovée JVMG, Cleton-Jansen AM, Kuipers-Dijkshoorn N, et al. Loss of heterozygosity and DNA ploidy point to a diverging genetic mechanism in the origin of peripheral and central chondrosarcoma. *Genes Chrom Cancer* 1999;**26**:237–46.
14. Baelde HJ, Cleton-Jansen AM, van Beerendonk H, et al. High quality RNA isolation from tumours with low cellularity and high extracellular matrix component for cDNA microarrays: application to chondrosarcoma. *J Clin Pathol* 2001;**54**:778–82.
15. Hameetman L, Rozeman LB, Lombaerts M, et al. Peripheral chondrosarcoma progression is accompanied by decreased Indian Hedgehog (IHH) signalling. *J Pathol* 2006;**209**:501–11.
16. Vandesompele J, De Preter K, Pattyn F, et al. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002:3. research0034.1–0034.11.
17. Gil-Benso R, Lopez-Gines C, Lopez-Guerrero JA, et al. Establishment and characterization of a continuous human chondrosarcoma cell line, ch-2879: comparative histologic and genetic studies with its tumor of origin. *Lab Invest* 2003;**83**:877–87.
18. Kunisada T, Miyazaki M, Mihara K, et al. A new human chondrosarcoma cell line (OUMS-27) that maintains chondrocytic differentiation. *Int J Cancer* 1998;**77**:854–9.
19. Kalinski T, Krueger S, Pelz AF, et al. Establishment and characterization of the permanent human cell line C3842 derived from a secondary chondrosarcoma in Ollier's disease. *Virchows Arch* 2005;**446**:287–99.
20. Cleton-Jansen AM, van Beerendonk HM, Baelde HJ, et al. Estrogen signaling is active in cartilaginous tumors: implications for antiestrogen therapy as treatment option of metastasized or irresectable chondrosarcoma. *Clin Cancer Res* 2005;**11**:8028–35.
21. Schrage YM, Briaire-de Bruijn IH, de Miranda NFCC, et al. Kinome profiling of chondrosarcoma reveals Src-pathway activity and dasatinib as option for treatment. *Cancer Res* 2009;**69**:6216–22.
22. de Heer P, Sandel MH, Guertens G, et al. Celecoxib inhibits growth of tumors in a syngeneic rat liver metastases model for colorectal cancer. *Cancer Chemother Pharmacol* 2008;**62**:811–9.
23. Grosch S, Maier TJ, Schiffmann S, Geisslinger G. Cyclooxygenase-2 (COX-2)-independent anticarcinogenic effects of selective COX-2 inhibitors. *J Natl Cancer Inst* 2006;**98**:736–47.
24. Su B, Diaz-Cruz ES, Landini S, Brueggemeier RW. Suppression of aromatase in human breast cells by a cyclooxygenase-2 inhibitor and its analog involves multiple mechanisms independent of cyclooxygenase-2 inhibition. *Steroids* 2008;**73**:104–11.
25. Solomon SD, McMurray JJ, Pfeffer MA, et al. Cardiovascular risk associated with celecoxib in a clinical trial for colorectal adenoma prevention. *New Engl J Med* 2005;**352**:1071–80.
26. Bresalier RS, Sandler RS, Quan H, et al. Cardiovascular events associated with rofecoxib in a colorectal adenoma chemoprevention trial. *New Engl J Med* 2005;**352**:1092–102.
27. Frampton JE, Keating GM. Celecoxib: a review of its use in the management of arthritis and acute pain. *Drugs* 2007;**67**:2433–72.