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Molecular characterisation of canine nonsteroidal anti-inflammatory drug-activated gene (*NAG-1*)

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Abstract

Nonsteroidal anti-inflammatory drug (NSAID)-activated gene (*NAG-1*), a divergent member of the transforming growth factor β superfamily, was previously identified as a gene induced by several anti-tumorigenic compounds, including NSAIDs and peroxisome proliferator-activated receptor γ (PPARγ) ligands in humans. In this study, canine *NAG-1* was characterised from a canine genomic database. Gene induction by some NSAIDs and PPARγ ligands was demonstrated in canine osteosarcoma cell lines. Phylogenetic analysis indicates that canine *NAG-1* is more homologous with the corresponding mouse and rat genes than with human *NAG-1*. Expression of canine *NAG-1* was increased by treatment with piroxicam and SC-560 (NSAIDs) and the PPARγ ligand rosiglitazone. This study demonstrates that canine *NAG-1* is up-regulated by some anti-tumorigenic compounds in osteosarcoma cell lines and may provide an important target of chemotherapy in canine cancer.

Keywords

NAG-1; NSAID; PPARγ ligand; TGF-β; Canine osteosarcoma

1. Introduction

Nonsteroidal anti-inflammatory drugs (NSAIDs) are widely used in the treatment of inflammatory disease. In addition, recent epidemiological studies in humans and experiments in spontaneous canine tumours and chemically-induced rodent tumours indicate that NSAIDs have anti-tumorigenic and chemotherapeutic effects in several different types of cancer (Reddy et al., 2000; Garcia Rodriguez and Huerta-Alvarez, 2001).

Treatment with the NSAID piroxicam had anti-tumorigenic activity against canine transitional cell carcinoma of the urinary bladder, accompanied by induction of tumour apoptosis and a reduction in angiogenic factor concentrations (Mohammed et al., 2003). A number of NSAIDs

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reduced cell proliferation rate, inhibited cell cycle progression and induced apoptosis in colorectal cancer cells (Goldberg et al., 1996, Baek et al., 2001; 2002b). Administration of the NSAID sulindac dramatically reduced the number of tumours in the intestinal tract of *Min* mice, a strain containing a fully penetrant dominant mutation in the *Apc* gene that leads to the development of gastrointestinal adenomas (Boolbol et al., 1996). Thus, NSAIDs provide many benefits, including anti-tumorigenic effects.

The transforming growth factor β (TGF-β) superfamily constitutes a group of related cytokines that have activity regulating cell survival, proliferation, differentiation, apoptosis, extracellular matrix formation and immunosuppression (Kingsley, 1994). Although TGF-β is referred to as the "molecular Jekyll and Hyde of cancer," TGF-β superfamily members act as potent growth suppressors via mechanisms including G_1 cell cycle arrest, induction of apoptosis and expression of cell adhesion molecules (Bierie and Moses, 2006).

Recently, we identified *NAG-1* (NSAID-activated gene), a member of the TGF-β superfamily, by polymerase chain reaction (PCR)-based subtractive hybridisation from an indomethacin-induced library in human colorectal cancer cells (Baek et al., 2001). Induction of *NAG-1*, subsequently, was observed following treatment with a number of other NSAIDs, including cyclooxygenase-1 (COX-1) specific, COX-2 specific and conventional NSAIDs (Baek et al., 2002b).

The protein encoded by *NAG-1*, which has been isolated by a variety of cloning strategies, is also known as macrophage inhibitory cytokine-1 (MIC-1) (Bootcov et al., 1997), placental transforming growth factor β (PTGFB) (Tan et al., 2000), prostate derived factor (PDF) (Paralkar et al., 1998), growth differentiation factor 15 (GDF15) (Hsiao et al., 2000) and placental bone morphogenetic protein (PLAB) (Hromas et al., 1997). The diversity of biological functions indicated by this nomenclature suggests that the biological role of the NAG-1 protein depends on cell context.

In several types of cancer cells, the NAG-1 protein is thought to play a role as a pro-apoptotic/anti-tumorigenic gene. For example, *NAG-1* is highly expressed in mature intestinal epithelial cells, but it is significantly reduced in human colorectal carcinoma samples and neoplastic intestinal polyps of *Min* mice (Kim et al., 2002). Furthermore, *NAG-1* overexpression can reduce MDA-MB-468 and MCF-7 breast cancer cell viability by up to 80% (Li et al., 2000). Treatment of prostate cancer cells with purified NAG-1 protein induces apoptosis (Liu et al., 2003). It has also been shown that *NAG-1* expression can be induced by p53 activation and that conditioned medium from cells overexpressing *NAG-1* suppresses tumour cell growth (Tan et al., 2000). These data support the link between *NAG-1*, apoptosis and tumour suppressing activity.

NAG-1 is up-regulated by a number of anti-tumorigenic compounds in addition to NSAIDs, including peroxisome proliferator-activated receptor γ (PPARγ) ligands (Baek et al., 2003; 2004b; Yamaguchi et al., 2006a), phosphatidylinositol 3-kinase inhibitor (Yamaguchi et al., 2004) and chemopreventive dietary compounds, such as resveratrol (Baek et al., 2002a), indole-3-carbinol (Lee et al., 2005), conjugated linoleic acid (Lee et al., 2006) and epicatechin gallate (Baek et al., 2004a), as well as anti-inflammatory plant extracts (Yamaguchi et al., 2006b). Interestingly, induction of *NAG-1* by these compounds occurs by multiple mechanisms at the levels of transcription and post-transcription. For instance, NSAIDs and resveratrol can transcriptionally activate *NAG-1* through early growth response-1 and p53, respectively (Baek et al., 2002a; 2005).

NAG-1 has been well-characterised in humans, but it has not been studied previously in dogs. Since NSAIDs have been used for treatment of some canine cancers, the function of canine NAG-1 protein should also be explored in canine cancer cells. In this report, we characterise

the canine *NAG-1* sequence, demonstrate the distribution of *NAG-1* expression in canine tissues and investigate canine *NAG-1* expression in response to NSAIDs and PPARγ ligands.

2. Materials and methods

2.1. Cells and reagents

Canine osteosarcoma cell line CCL-183 was purchased from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 μg/mL), sodium pyruvate and L-glutamine (2 mM). NSAIDs used in this study were purchased as follows: [5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl) phenyl-2 (*5H*)-furanonel] (DFU) was purchased from Merck, sulindac sulphide and SC-560 were from Cayman Chemical Company and diclofenac and piroxicam were from Sigma. PPARγ ligands rosiglitazone and MCC-555 were purchased from Cayman Chemical Company. All compounds were dissolved in dimethyl sulphoxide (DMSO).

2.2. Preparation of RNA and semi-quantitative reverse transcriptase-PCR

Total RNA was extracted from CCL-183 cells using TRIzol reagent (Invitrogen). One microgram of total RNA was reverse-transcribed to produce single strand cDNAs using an iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. On the basis of the predicted cDNA sequence of canine *NAG-1* (GenBank XM_541938), primers were synthesised as follows: sense, 5′-gcacctgcagaccaagctga-3′; antisense, 5′-cgtccgggaacctacacgac-3′. REDTaq ReadyMix PCR Reaction Mixture (Sigma) was used for PCR. The thermocycling conditions for amplification of canine *NAG-1*, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and *COX-2* genes were 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min.

Primer sequences used for canine *GAPDH* (GenBank NM_001003142) were: sense, 5′-gtgtccccacccccaatgta-3′; antisense, 5′-acccggttgctgtagccaaa-3. ′ For canine *COX-2* (NM_001003354) the primer sequences are sense, 5′-aacacctgcagtttgctgtg-3′; antisense 5′-gcagctctgggtcaaacttc-3′. The final PCR products were electrophoresed on 1.4% agarose gels and photographed under ultraviolet light. Reverse transcriptase PCR (RT-PCR) for *GAPDH* was used as an equal loading control.

2.3. Western blot analysis

Tissue samples were obtained from a research animal from another study that was euthanased in accordance with the institutional animal care and use committee protocol. Tissue lysates were prepared from samples stored at −70 °C by homogenisation in radioimmunoprecipitation assay (RIPA) buffer containing 1x phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate (SDS), supplemented with protease and phosphatase inhibitors (1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mM phenylmethylsulphonyl fluoride, 0.1 mM Na_3VO_4 and 25 mM NaF). Samples were sonicated three times for 10 s, then centrifuged for 30 min at 12,000 *g* to remove debris and the supernatants were used for analysis.

Protein concentrations were determined by the bicinchronic acid (BCA) method using BCA protein assay reagent (Pierce) and bovine serum albumin as a standard. Samples were boiled in Laemmli sample buffer, electrophoresed on 16% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Osmonics). Blots were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T). The blots were incubated for 1 h at room temperature with a polyclonal anti-human NAG-1 antibody (1:1,000 dilution) (Baek et al., 2001), in 5% skim milk in TBS-T.

To examine whether the signal is specific to the canine NAG-1 protein band, the antibody was mixed with NAG-1 peptide (1 mg/mL) for 4 h at 4 °C and then followed by incubation with NAG-1 antibody as described. After washing, the blots were incubated with anti-rabbit horseradish peroxidase conjugated secondary antibody for 1 h and washed again. The signal was detected by the enhanced chemiluminescence system (Amersham).

2.4. Sequence analysis

The deduced amino acid sequence of the canine NAG-1 protein was generated by DIALIGN 2.2.1 (http://bibiserv.techfak.uni-bielefeld.de/dialign/). The sequence results were compared with those of other species within the National Center for Biomedical Information's GenBank database and multiple alignments were performed by the AliBee program (GeneBee, http://www.genebee.msu.su/).

3. Results

3.1. Comparisons of predicted canine NAG-1 sequences with other species and molecular phylogenetic analysis

The deduced amino acid sequence of the canine NAG-1 protein was generated by DIALIGN 2.2.1 (Fig. 1). The full-length canine *NAG-1* cDNA has 924 bp and 307 deduced amino acids. The deduced amino acid sequence of the NAG-1 protein from other species was obtained from the GenBank database and multiple alignments were generated by the AliBee program. This alignment shows nine conserved cysteine residues in the C-terminal end and an RXXR motif (Fig. 2a). As shown in Fig. 2b, phylogenetic analysis indicated the highest degree of homology between the canine NAG-1 protein and mouse and rat NAG-1.

3.2. Tissue distribution of canine NAG-1

To investigate the tissue distribution of the canine NAG-1 protein, Western blot analysis was performed using canine kidney, skeletal muscle, jejunum, lung, spleen, heart and liver using a polyclonal anti-human NAG-1 antibody. Strong bands were observed in kidney, lung and liver (Fig. 3a). High-level expression of NAG-1 protein in liver is also seen in the mouse (Hsiao et al., 2000a).

We also determined whether human NAG-1 antibody (Baek et al., 2001) specifically recognises canine NAG-1 protein. As shown in Fig. 3b, incubation of NAG-1 peptide with the antibody markedly attenuated the detection of specific bands (~35 kDa), demonstrating that anti-human NAG-1 antibody recognises canine NAG-1 protein. Interestingly, the intensity of an unexpected band (~60 kDa) seen in skeletal muscle was also diminished. This band probably represents pro-NAG-1 hemidimer (Bauskin et al., 2000).

3.3. Effects of NSAIDs and PPARγ ligands on NAG-1 expression in CCL-183

NAG-1 induction by NSAIDs is well established in human colorectal cancer cell lines, as well as osteosarcoma cell lines (Baek et al., 2002a). Based on this information, we sought to analyse *NAG-1* induction in canine cancer cells. Canine osteosarcoma CCL-183 cells were treated with DMSO or NSAIDs (DFU, diclofenac, piroxicam, SC-560 and sulindac sulphide) to determine if these compounds induce *NAG-1* in canine osteosarcoma cells. Plasmid containing canine *NAG-1* cDNA was used as a positive RT-PCR control. As shown in Fig. 4a, *NAG-1* induction was evident in piroxicam and SC-560 treated cells.

COX-2 expression was also analysed because its expression has been known to be repressed by some NSAIDs (Xu et al., 1999). Treatment with DFU reduced *COX-2* expression when compared to the vehicle, but other NSAIDs did not affect *COX-2* expression.

PPARγ ligands have been characterised as having anti-tumorigenic effects in numerous types of human cancer showing induction of apoptosis and *NAG-1* expression (Yamaguchi et al., 2006a). To see if the same phenomenon occurred in canine osteosarcoma, CCL-183 cells were treated with MCC-555 and rosiglitazone, both of which induce *NAG-1* expression in human colorectal cancer cells. As shown in Fig. 4b, treatment with rosiglitazone induced *NAG-1* expression, but treatment with MCC-555 did not.

4. Discussion

In the last decade, studies with microarray and PCR-based subtractive hybridisation have identified a number of genes that are targeted by anti-tumorigenic compounds. *NAG-1* was identified from an indomethacin-induced gene library in our laboratory (Baek and Eling, 2006). Human *NAG-1* encodes a protein with homology to members of the TGF-β superfamily. Intra- and inter-molecular bonds formed by six to nine conserved cysteine residues create a structure characteristic of the TGF-β superfamily.

NAG-1 is a dimeric secreted protein consisting of a long propeptide separated from the mature protein by furin-like proconvertase at a conserved RXXR sequence (Bauskin et al., 2000). Based on GenBank database comparison, the predicted canine NAG-1 amino acid sequence has nine cysteine residues and an RXXR sequence conserved with human, mouse, rat and chimpanzee NAG-1, suggesting that canine NAG-1 protein has a biological activity (Fig. 2).

It has been known that the human *NAG-1* transcript is highly expressed in placenta and at lower levels in the colon, kidney and prostate (Paralkar et al., 1998a). Canine *NAG-1* is highly expressed in the liver and lung, whereas human *NAG-1* is not expressed in liver (Fig. 3). On the other hand, mouse *NAG-1* is highly expressed in the liver and expressed at moderate levels in the kidney (Hsiao et al., 2000a), indicating a different distribution of basal *NAG-1* expression among species. However, phylogenetic tree analysis (Fig. 2b) indicates that canine *NAG-1* is more closely related to mouse *NAG-1* than the corresponding human molecule in tissue distribution.

NSAIDs represent a promising approach to cancer therapy (Ulrich et al., 2006) because they inhibit COX-1 and/or COX-2, which are responsible for the formation of prostaglandins from arachidonic acid. Overexpression of COX-2 is often observed in many types of cancer cells and results in an increase of cell survival (Lin et al., 2001) and metastasis (Tsujii et al., 1997) and a reduction of apoptosis (Nzeako et al., 2002). COX inhibition by NSAIDs at the protein level is likely to be closely related to their anti-tumorigenic effects.

However, NSAIDs can also act through COX-independent mechanisms (Grosch et al., 2006). Induction of canine *NAG-1* was observed only in the presence of piroxicam or SC-560. On the other hand, the NSAIDs diclofenac and sulindac sulphide did not induce *NAG-1*. Furthermore, *NAG-1* induction was not observed in the presence of the COX-2 specific inhibitor DFU (Fig. 4). These data suggest that COX inhibition does not necessarily increase *NAG-1* expression and a COX-independent pathway may be involved in *NAG-1* induction by NSAID.

Several studies demonstrated that the PPARγ ligand rosiglitazone inhibits cell growth (Brockman et al., 1998). Rosiglitazone also markedly induced *NAG-1* expression. Since canine *NAG-1* is induced by both NSAID and PPARγ ligand treatment, it can be inferred that *NAG-1* induction occurs via several mechanistic pathways.

5. Conclusion

The canine *NAG-1* gene is more homologous with mouse and rat *NAG-1*, than with the corresponding human gene. Some NSAIDs and rosiglitazone up-regulate canine *NAG-1*,

consistent with the notion that the NAG-1 protein may play an important role as a mediator for chemotherapeutic agents in canine cancer. Further studies will be required to elucidate the pathways for *NAG-1* induction by NSAID and PPARγ ligands in dogs.

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  1 atg cct gga cag gga ccg gca cca gca cac tgc tct ccc atg ctg gtg ata ctg gtg atg
    M   P   G   Q   G   P   A   P   A   H   C   S   P   M   L   V   I   L   V   M
 61 ctc tcc tgg ctg cct tcg gga ggc gcc ctg tct ctg gcc cag gag cac ctc ccc gcc ttt
    L   S   W   L   P   S   G   G   A   L   S   L   A   Q   E   H   L   P   A   F
121 cca gga ccc tca gac ccg cac tcc agt acc gac gtg tcc aga atc cag gag ctt cgg aaa
    P   G   P   S   D   P   H   S   S   T   D   V   S   R   I   Q   E   L   R   K
181 cgc tac gag cac ctg cag acc aag ctg agg ctg aac caa ggc tgg gcc gat tca aac cct
    R   Y   E   H   L   Q   T   K   L   R   L   N   Q   G   W   A   D   S   N   P
241 gac ctc gtc cct gca act cga gtc cgg ata ctc act cca aag ctg cga ctt ggg ccg cga
    D   L   V   P   A   T   R   V   R   I   L   T   P   K   L   R   L   G   P   R
301 ggc cac ctg cac ctg cgc atc gcc cgg gcc gac ctg act gcg ggg ctc ccc gca gcc tcc
    G   H   L   H   L   R   I   A   R   A   D   L   T   A   G   L   P   A   A   S
361 cgc cta cat cgg gcg ctg ctc aga ttg tcc ccg acg gag ccg agc tcg tgg gac gtg acg
    R   L   H   R   A   L   L   R   L   S   P   T   E   P   S   S   W   D   V   T
421 cgg ccg ctg cag cgt cag ctc agt cgt gta ggt tcc cgg acg ccc aca cta cgc ctg cga
    R   P   L   Q   R   Q   L   S   R   V   G   S   R   T   P   T   L   R   L   R
481 ctg ttg cct cgg tgg gac cgg tcg cga gcg ttg cct tct gca cgt ccg cag ctt gag ctg
    L   L   P   R   W   D   R   S   R   A   L   P   S   A   R   P   Q   L   E   L
541 cac tgg cgg cca cgc gcg gcc agg ggg cgc cgc aac gcg cat gcg cac gcc cgg gac ggt
    H   W   R   P   R   A   A   R   G   R   R   N   A   H   A   H   A   R   D   G
601 tgc ccc ctc ggg gag ggg cgc tgc tgc cgc ctg caa agc ctg cgc gcg tcg ctc cag gac
    C   P   L   G   E   G   R   C   C   R   L   Q   S   L   R   A   S   L   Q   D
661 ctg ggt tgg gcc aac tgg gtg gtg gcg ccc cgc gag ctg gac gtg cgc atg tgt gta ggc
    L   G   W   A   N   W   V   V   A   P   R   E   L   D   V   R   M   C   V   G
721 gcg tgc cca agc cag ttc cgg tcg gct aac acg cac gcc cag atg cag gcg cgc ctg cat
    A   C   P   S   Q   F   R   S   A   N   T   H   A   Q   M   Q   A   R   L   H
781 ggc ctg aac ccc gac gcc gcg cct gcg ccg tgc tgc gtg ccc gcc agc tat gag ccg gtg
    G   L   N   P   D   A   A   P   A   P   C   C   V   P   A   S   Y   E   P   V
841 gtg ctc atg cac caa gac agc gac ggg cgc gtg tcg ctg acg ccc ttc gac gac ctc gtg
    V   L   M   H   Q   D   S   D   G   R   V   S   L   T   P   F   D   D   L   V
901 gcc aag gac tgc cac tgc gtg tga
    A   K   D   C   H   C   V   *
```

Fig. 1.
Predicted nucleotide sequence (top) and deduced amino acid sequence (bottom) of canine *NAG-1* **(**GenBank XM_541938).

```
Dog              1 M--------PGQGPAPAHCSPMLVILVMLSWLPSGGALSLAQEHLPAFPGPSDPHSSTDV
Human            1 M--PGQELRTVNGSQ------MLLVLLVLSWLPHGGALSLAEASRASFPGPSELHS--ED
Mouse            1 MAPPALQAQPPGGSQLRFLLFLLLLLLLLSWPSQGDALAMPEQRRSGPESQLNA------
Rat              1 MALRALHAQPTGGPQLRFLLFLLLLLLLLSWPSQGDALALPEQRRSLSESQLNP------
Chimpanzee       1 M---GSVARQRGGTAAPD---VAMGVVVLSTPSY----ELSPW-RPGPPVERRILS----

Dog             53 SRIQELRKRYEHLQTKL-RLNQGWADSNPDLVPATRVRILTPKLRLGPRGHLHLRIARAD
Human           51 SRFRELRKRYEDLLTRL-RANQSWEDSNTDLVPAPAVRILTPEVRLGSGGHLHLRISRAA
Mouse           55 ---DELRGRFQDLLSRL-HANQSREDSNSEPSPDPAVRILSPEVRLGSHGQLLLRVNRAS
Rat             55 ---DELRGRFQDLLSRL-HANQSREDSNSEPTPDPAVRILSPEVRLGSHGRLLLRVNRAS
Chimpanzee      46 ------AFDFSFLICKTpHQRKTPPTSGLELQGFGA-----GTMRLGSGGHLHLRISRAA

Dog            112 LTAGLPAASRLHRALLRLSPTEPSSWDVTRPLQRQLSRVGSRTPTLRLRLLPRWDRSR--
Human          110 LPEGLPEASRLHRALFRLSPTASRSWDVTRPLRRQLSLARPQAPALHLRLSPPPSQSDQL
Mouse          111 LSQGLPEAYRVHRALLLLTPTT-RPWDITRPLKRALSLQGPRAPALRLRLTPPPDLAM--
Rat            111 LTQGLPEAYRVHRALLLLTPSS-RPWDITRPLQRAISLQGPHARALRLRLAPPPDLAV--
Chimpanzee      95 LPEGLPEASRLHRALFRLSPTAARSWDVTRPLRRQLSLARPQAPALHLRLSPPPSQSDQL

                                                  *       **
Dog            170 ALP--SARPQLELHWRPRAARGRRNAHAHARDGCPLGEGRCCRLQSLRASLQDLGWANWV
Human          170 LAESSSARPQLELHLRPQAARGRRRARARNGDDCPLGPGRCCRLHTVRASLEDLGWADWV
Mouse          168 -LP--SGGAQLELRLRVAAGRGRRSAHAHPRDSCPLGPGRCCHLETVQATLEDLGWSDWV
Rat            168 -LP--SGGARLELHLRSAAGRGRRSAHLHPRDSCPLGPGRCCHLETVQATLEDLGWSDWV
Chimpanzee     155 LAESSSARPQLELHLRSRAARGLRRARARNGDHCPLGPGRCCRLHTVRASLEDLGWSDWV

                     *   *                                  **
Dog            228 VAPRELDVRMCVGACPSQFRSANTHAQMQARLHGLNPDAAPAPCCVPASYEPVVLMHQDS
Human          230 LSPREVQVTMCIGACPSQFRAANMHAQIKTSLHRLKPDTEPAPCCVPASYNPMVLIQKTD
Mouse          225 LSPRQLQLSMCVGECPHLYRSANTHAQIKARLHGLQPDKVPAPCCVPSSYTPVVLMHRTD
Rat            225 LSPRQLQLSMCVGECPHLYRSANTHALIKARLHGLQPDRVPAPCCVPSSYTPVVLMHRTD
Chimpanzee     215 LSPREVQVTMCIGACPSQFRAANMHAQIKTSLHRLKPDTVPAPCCVPASYNPMVLIQKTD

                                 * *
Dog            288 DGRVSLTPFDDLVAKDCHCV
Human          290 TG-VSLQTYDDLLAKDCHCI
Mouse          285 SG-VSLQTYDDLVARGCHCA
Rat            285 SG-VSLQTYDDLVAQGCHCA
Chimpanzee     275 TG-VSLQTYDDLLAKDCHCI
```

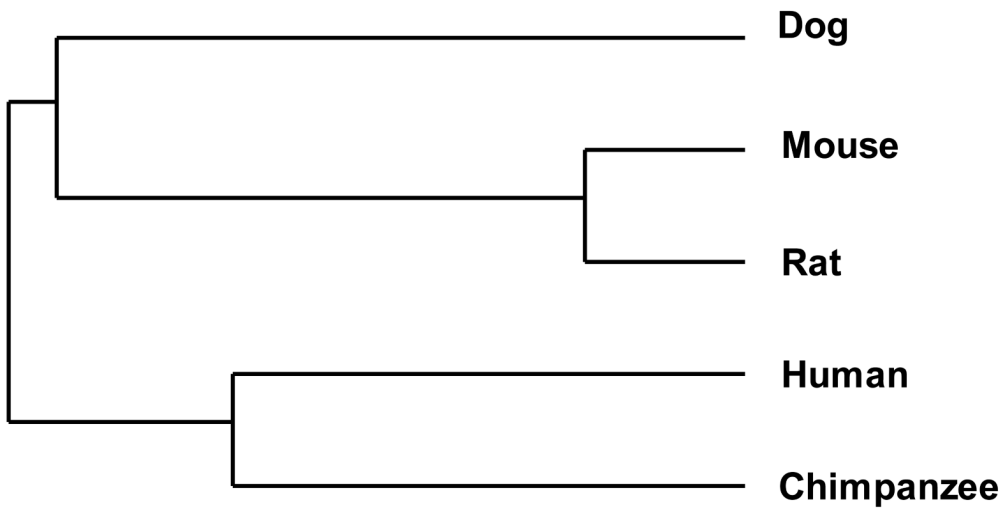

Fig. 2.

Alignment of NAG-1 amino acid sequence from the dog, human, mouse, rat, and chimpanzee (GenBank: human, AAH08962; mouse, NP_035949; rat, CAA09891; chimpanzee, XP_524157). (a) Amino acid sequence alignment of the C-terminal region begins with the first conserved cysteine residue. Conserved cysteine residues are marked with an asterisk (*). Bold letter indicates RXXR motifs. (b) Phylogenetic tree based on NAG-1 amino acid sequence homology among the dog, human, mouse, rat, and chimpanzee.

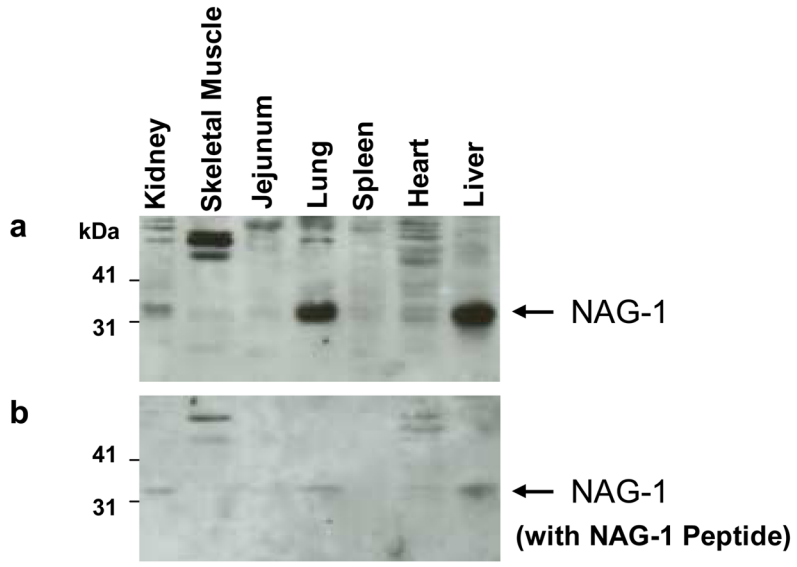

Fig. 3.

Expression profiles of NAG-1 protein in canine tissues demonstrated by Western blot analysis using 30 μg of cell lysate from each tissue separated by SDS-PAGE. (a) Polyclonal anti-human NAG-1 antibody. Canine NAG-1 appeared as a band ~ 35 kDa. (b) Result from incubating NAG-1 antibody with human NAG-1 peptide.

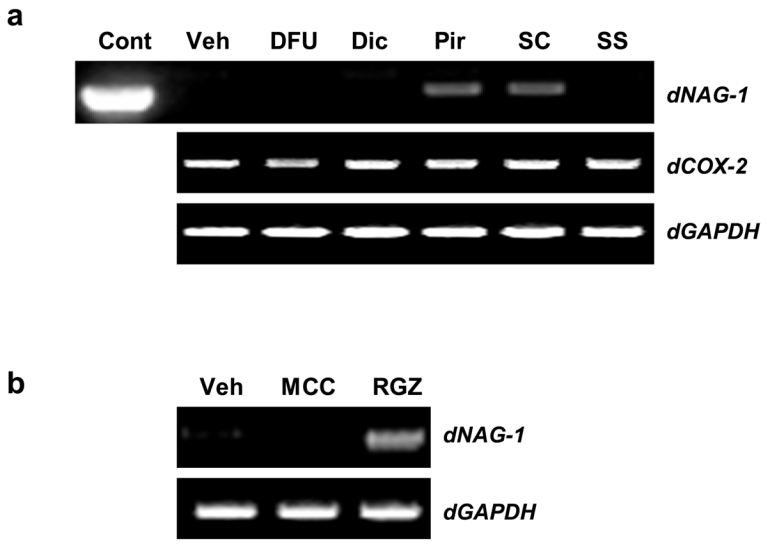

Fig. 4.

Effects of NSAID and PPARγ ligands on *NAG-1* expression in canine osteosarcoma cancer cells determined by RT-PCR. (a) CCL-183 cells were treated for 24 h with DMSO vehicle (Veh) or NSAIDs: DFU: 5,5-dimethyl-3-(3-fluorophenyl)-4-(4-methylsulphonyl) phenyl-2 (*5H*)-furanonel, 100 μM; Dic: diclofenac, 100 μM; Pir: piroxicam, 100 μM; SC: SC-560, 50 μM; SS: sulindac sulphide, 30 μM. Plasmid containing canine *NAG-1* cDNA was used as a positive control (Cont). (b) CCL-183 cells were treated for 24 h with vehicle (Veh) or PPARγ ligands: MCC: MCC-555, 5 μM; RGZ: rosiglitazone, 5 μM. RT-PCR was performed using total RNA isolated from cells. Equal loading was demonstrated by GAPDH RT-PCR.