Fibrodysplasia Induced in Dog Skin by a Matrix Metalloproteinase (MMP) Inhibitor—A Mechanistic Analysis

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Matrix metalloproteinase (MMP) inhibitors, candidate therapeutic agents for a number of diseases, are known to be associated with acute fibrosis-type adverse effects in a number of species, including humans. The broad-spectrum MMP inhibitor, AZM551248, has previously been shown to cause these effects in the dog. Changes were characterized by the abnormal and extensive proliferation of fibroblasts and the deposition of collagen particularly in the subcutaneous connective tissues (subcutis) and were termed fibrodysplasia (FD). We performed a time-course study in dogs using AZM551248 and sampled skin, subcutis, and plasma before and during the development of FD. Detailed histopathological analysis and global gene expression profiling were performed on the subcutaneous tissues. The gene expression analysis of the subcutis indicated that extracellular matrix (ECM) remodeling was initiated asymptotically at or before the earliest time point, day 4, and this was associated with dysregulation of expression of a number of MMPs and proteolytic enzymes. At later time points, the FD became progressively more extensive and severe, and this was associated with gene expression changes characteristic of tissue fibrosis, for example those associated with procollagen synthesis and processing. We postulate that AZM551248 inhibition of MMP action within the subcutis modulates the activity of several transcription factors and this in turn upregulates expression of specific proteases which initiate ECM remodeling. Persistent MMP inhibition results in the progression of ECM remodeling, culminating in collagen deposition and overt fibrosis. Our data indicate that inhibition of MMPs 1, 2, 3, and 9 is a key early event in AZM551248-induced FD in dog subcutis.

Key Words: matrix metalloproteinases; fibrosis; gene expression; ECM remodeling.

Matrix metalloproteinases (MMPs) are a diverse family of zinc-dependent proteolytic enzymes that are involved in the maintenance of the extracellular matrix in normal physiology and that play important roles in ECM breakdown in pathological and disease situations (Brinckerhoff and Matrisian, 2002; Murphy et al., 2002; Visse and Nagase, 2003). The MMPs have been investigated as potential therapeutic targets for a number of diseases, including osteoarthritis (OA), rheumatoid arthritis, osteoporosis, tumor metastasis, and periodontal disease (Clark and Parker, 2003; Coussens et al., 2002; Overall and Kleifeld, 2006). In particular, MMP 13 or collagenase 3, has often been quoted as the target of choice for OA therapy, because MMP 13 expression appears to be restricted to pathological tissue, and the enzyme and its substrate cleavage products are found in human OA tissue (Konttinen et al., 1999; Wernicke et al., 2006; Westhoff et al., 1999; Wu et al., 2002).

To date, the development of a number of MMP inhibitors (MMPis) has been hampered by the occurrence of connective tissue pathologies both preclinically and clinically. These can manifest as musculoskeletal syndrome (MSS) in man (Drummond et al., 1999; King et al., 2003; Krzeski et al., 2007; Peterson, 2006). MSS is characterized by joint pain initially in the extremities, which can spread to other joints and cause reduced mobility (“frozen shoulder”). In some cases, subcutaneous skin thickening in the hands occurs, accompanied by progressive contracture of the digits (Tierney et al., 1999). This has been likened to Dupuytren’s disease, a genetic condition in which thick subcutaneous fibrous material is deposited, leading to pain and deformation of the finger joints (Hutchinson et al., 1998). In toxicology studies with MMPis in rats, clinical signs, particularly hind paw swelling and altered movement, are associated with several characteristic histopathological changes, including collagen synthesis and remodeling in the joint spaces with lymphocyte infiltration, myofibroblast proliferation, and fusion of the synovium, muscle, and tendon tissue (Renkiewicz et al., 2003). These features have been collectively termed fibrodyssplasia (FD) (Westwood et al., 2009).

In the dog, FD induced by an MMPi is manifest initially in the skin subcutis, although musculoskeletal tissues are also affected (Westwood et al., 2009). FD is a significant adverse finding associated with MMPi treatment in a number of preclinical test species and is considered consistent with the clinical findings of MSS that has been observed in humans treated with these compounds.
However, the compounds that have been shown to be associated with FD in animal safety models, and with MSS development in man, are “broad spectrum” MMPis, i.e., they exhibit inhibitory activity against several MMP family members. It has been suggested that although a broad spectrum of MMP inhibitory activity may be beneficial in terms of improved disease modification, this may be an important factor in the onset of FD and MSS (Peterson, 2006). This is supported by recent evidence that more selective MMP 13 inhibitors do not cause joint FD in the rat, albeit in dosing studies of 14 days duration (Baragi et al., 2009; Johnson et al., 2007).

In the experiments reported here, we undertake a molecular study of subcutaneous FD in the dog associated with a broad-spectrum prototype small molecule MMPi, AZM551248 (Fig. 1). The aims of the study were (1) to associate the pathological stages of FD development in the dog subcutis with key biochemical pathway changes and (2) to identify early gene expression markers in the subcutis, which occur before the onset of pathological FD, thereby illuminating the mechanism of the AZM551248-induced changes.

**MATERIALS AND METHODS**

**AZM551248 in vivo study.** The AZM551248 time-course study design and resulting subcutaneous pathological changes have been reported in detail elsewhere (Westwood et al., 2009). Briefly, 30 female beagle dogs, around 12 months of age, were assigned to six treatment groups of five animals as outlined in Table 1. Animals were dosed orally once daily either with vehicle alone (0.5% wt/vol hydroxypropyl methylcellulose/0.1% polysorbate 80, 5 ml/kg) or with vehicle plus 20 mg/kg/day AZM551248. This dose of AZM551248 had previously been shown to cause subcutaneous FD after 17 days in a small pilot study where two female beagle dogs were dosed over a 25-day period with 20 mg/kg/day AZM551248 (data not shown). At each necropsy time, a histopathological analysis was carried out on subcutaneous tissue, dermal skin, synovium, tendon, and muscle from several sites. In addition, dermal and subcutaneous tissue samples from the left and right abdomen, left and right dorsal cervical and lumbar regions, and adjacent to the calcaneal tendon and gastrocnemius muscle with associated tendon were also taken. These samples were snap frozen in liquid nitrogen for RNA analysis; the analysis described herein is confined to the dorsal cervical subcutaneous skin. The details of the histopathological analysis are described elsewhere (Westwood et al., 2009).

**Determination of TGF-β in plasma.** Blood was taken from the two animals in the pilot study, from 6 days prior to the onset of dosing until day 25, and plasma prepared. Levels of transforming growth factor-β (TGF-β) were determined in plasma samples by immunoassay according to the manufacturer’s instructions (Cat No. MB100B; R&D Systems, Abingdon, U.K.). This was determined based on publicly available canine sequence information: MMP 1, MMP 3, MMP 9, MMP 13, DPP4, ANPEP, and CatK. Custom TaqMan Gene Expression Assays were designed for the noninventoried assays: MMP 2, MMP 3, MMP 9, MMP 13, DPP4, ANPEP, and CatK. TaqMan Gene Expression Assays (Applied Biosystems) and 2X PCR MasterMix (Eurogentec, RT-QP2X-03+WOULR [low ROX]) in a total reaction volume of 20 μl. Amplification was performed using a Stratagene MX4000 cycler, with an initial denaturation step of 10 min at 95°C followed by 45 s at 60°C.

**RNA preparation.** Total RNA was isolated from dorsal cervical subcutaneous skin tissue using the RNeasy lipid tissue midi kit (Qiagen), following the manufacturer’s instructions. RNA was quantified using a NanoDrop ND-1000 spectrophotometer (ThermoFisher), and quality assessed using the Agilent RNA 6000 Nano Kit (5067-1511) and following the manufacturer’s standard protocol. Only RNA samples with an RNA integrity number of > 6 were used in subsequent analyses.

**Complementary RNA preparation and array hybridization.** Biotin-labeled, fragmented complementary RNA (cRNA) was prepared and purified using the Affymetrix One-Cycle Eukaryotic Target Labeling Assay according to the manufacturer’s instructions. Fifteen micrograms of cRNA was used in each hybridization reaction, using the GeneChip Canine Genome 2.0 Arrays (Affymetrix). Hybridization was carried out overnight at 45°C in a hybridization oven, and GeneChips were subsequently washed and stained according to manufacturer’s protocols, using Affymetrix fluidics stations.

**Array analysis.** Arrays were scanned using an Affymetrix 3000 scanner, and images quantified using Affymetrix GCOS software. Raw data were analyzed using the MA55 algorithm in order to score the probe set intensity and presence/absence call. MA55 scores for all chips were compiled into MS Excel spreadsheets for further analysis. Affymetrix probesets were annotated using a combination of NetAffx (Affymetrix) and proprietary in-house annotation.

**Bioinformatic analysis.** The expression of probesets was deemed to be significantly different between groups when the mean expression differed by > twofold, with a p value of < 0.05 (Student’s two-tailed t-test), and the probesets were called as present in the majority of samples in at least one group. A core set of dysregulated probesets (i.e., differentially expressed between the control [vehicle treated] group and one or more treated groups) was identified. The dysregulated probesets were subjected to principle component analysis (PCA) analysis using Spotfire software. Pathway analysis was also carried out, using GeneGo software.

**Quantitative RT-PCR analysis.** Complementary DNA (cDNA) was synthesized from 1 μg of total RNA, using Superscript III Reverse Transcriptase and random hexamers in a total volume of 20 μl as per manufacturer’s instructions (Invitrogen). Three microliters of template cDNA was added to a total reaction volume of 20 μl. Amplification was performed using a Stratagene MX4000 cycler, with an initial denaturation step of 10 min at 95°C followed by 40 cycles of 15 s at 95°C, 45 s at 60°C.

The following were obtained from Applied Biosystems as inventoried or noninventoried assays: MMP 2, MMP 3, MMP 9, MMP 13, DPP4, ANPEP, and CatK. Custom TaqMan Gene Expression Assays were designed for the following, based on publicly available canine sequence information: MMP 1, MMP 8, and glyceraldehyde 3-dehydrogenase (GAPDH).

**Determination of MMP inhibition by AZM551248.** This was determined using Quench Fluorescence Resonance Emission Transfer. Briefly, a peptide substrate was incubated with purified human enzyme at or near the Km, together with a range of concentrations of AZM551248. The peptide substrate M-1895

![FIG. 1. The structure of AZM551248.](image-url)
was used for each MMP.

The same peptide substrate (Mca-Pro-Leu-Gly-Leu-Dap[Dnp]-Ala-Arg-NH₂) was used for each MMP.

RESULTS

In Vivo Histopathological Analysis

The histopathological changes observed in the AZM551248 time-course study have been discussed in detail elsewhere (Westwood et al., 2009). The histological characteristics of the changes are illustrated in Figure 2, and the incidence and severity in the subcutaneous tissues summarized in Table 2. Briefly, no changes were observed in animals treated for 4 days but after 8 days treatment one animal showed fibroprotectic changes in the dorsal cervical subcutaneous tissues and one in the lumbar and dorsal cervical subcutaneous tissues. These changes were manifest as proliferation of enlarged cells showing myofibroblast differentiation, coupled with deposition of collagen fibers. At the day 11 time point and beyond, the FD changes progressed to greater incidence and severity, with increased cellularity and more extensive and abundant collagen deposition. In addition, there was a greater range of tissue involvement at the later time points, with effects specifically in the skin dermis, and the joint synovia, tendons, and ligaments. For the purposes of the current investigation, the focus is on the subcutaneous tissue as this is where evidence of FD was first noted, i.e., the tissue most sensitive to AZM551248-induced changes.

FIG. 2. Typical appearance of the dorsal cervical subcutaneous tissue of a control dog (animal 4) (A) and a dog dosed with AZM551248 for 11 days (animal 19) (B). In the control tissue, fibroblasts (thick arrows) are small and sparse. Collagen (thin arrows) is condensed but irregular in arrangement. The fibroprotectic tissue illustrated in (B) contains abundant enlarged fibroblast-like cells (thick arrows) and compact arrays of collagen showing a more fibrillar character (thin arrows). Bar = 100 µm.

Gene Expression Analysis

Total subcutaneous tissue RNA samples from each of the 30 animals in the study were analyzed using Affymetrix Canine Genome 2.0 arrays. The mean expression of each gene in each group of five animals was calculated, together with the p value for the difference in expression between the test group and corresponding control.

The number of significant (p < 0.05) gene expression changes relative to vehicle control for all time points is given in Table 2. A complete list of all significant gene expression changes, at all time points, is provided as Supplementary Data. It is clear that fewer changes occurred at earlier time points for all tissues, particularly day 4, consistent with the lack of histopathological changes at this time point. Many more changes occurred at the later time points, particularly day 14 and 17, concurrent with extensive tissue remodeling.

Despite of the lack of pathological changes in subcutaneous tissues at day 4, and minimal changes at day 8, all treatment groups could be distinguished from the vehicle group by PCA of the gene expression data set (Fig. 3). In particular, the PCA plot highlights a progression of the time course through PCA “space,” with change in trajectory coincident with the onset of pathology. Taken together, these data imply phased changes in gene expression induced by AZM551248; potentially causative changes occurring at early time points, and many more changes at later time points that reflect frank pathology.

To interrogate the relationship between the pharmacological action of AZM551248 and the observed gene expression changes, and to complement the array data set, we performed RT-PCR analysis to quantify the transcript changes associated with the MMPs inhibited by AZM551248 (Fig. 4). This revealed a transcriptional upregulation of MMPs 1, 2, 3, 9, and 13 at day 4, presumably compensatory increases in response to the inhibition of the activity of the corresponding proteases.

Next, we used GeneGo pathway analysis to identify the dysregulated biochemical/molecular pathways associated with AZM551248 treatment at day 4. Significantly, the top pathway “hits” were cell adhesion pathways and ECM remodeling (Fig. 5A), consistent with the known role of extracellular MMPs in these processes (Fig. 5B). Additionally, we focused on the transcription regulatory networks associated with the transcripts dysregulated by AZM551248 at day 4. The rationale is that upregulation of transcription factor activity, as a consequence of MMP inhibition, is driving the expression of genes that trigger the fibroprotectic response. Inevitably, some genes will be dysregulated as a “bystander” effect, but the assumption of this analysis is that those genes associated with the most affected transcription factors are the most functionally relevant.

Although most genes are regulated by multiple transcription factors, this approach pointed to the involvement of Sp1, RelA, and STAT1 as important regulators of the changes associated with AZM551248 treatment at day 4. It can be seen that these...
Factors are known to be involved in the regulation of MMPs 1, 2, and 3 (stromelysin-1), consistent with the inhibitory profile of AZM551248 (Fig. 5C). Therefore, the data suggest that these transcription factors increase activity in response to AZM551248 treatment. Although we observe many transcripts to be downregulated at early time points (Table 2), this is consistent with an increase in transcription factor activity, because these factors can stimulate or inhibit transcription depending on gene promoter structure and cellular context (Chu and Ferro, 2005).

It is known that other proteases besides MMPs are involved in ECM remodeling, and in this regard, it is interesting to note that the peptidases dipeptidyl peptidase IV (DPP4), the aminopeptidase ANPEP, and Cathepsin K are all upregulated at day 4. This was confirmed by RT-PCR analysis (Fig. 6), and notably, these genes are also under the control of Sp1, RelA, and STAT1 (Fig. 5C). Upregulation of Cathepsin K, DPP4, and ANPEP are maintained at day 8, where the first signs of pathological changes in the subcutaneous tissues are detectable in two of five animals. Interestingly, the one animal that showed moderate FD at day 8 in the cervical subcutis also showed the highest expression of DPP4, ANPEP, and CatK in this tissue at this time point (Fig. 6). At other time points, there was also a good correlation between expression of these three proteases and severity of pathology. Minimal pathological changes, characterized by the appearance of local proliferations of fibroblast-like cells, were noted in particular animals together with some deposition of collagen fibers (Westwood et al., 2009; Table 2). Such changes are very reminiscent of the epithelial-mesenchymal transition changes associated with the early stages of tissue fibrosis (Zeisberg and Neilson, 2009).

Shortly afterward, at day 11, the subcutaneous tissues began to show more extensive and severe manifestations of FD, specifically more abundant collagen deposition and proliferation of enlarged active fibroblasts, and multifocal mononuclear cell infiltrates. At this time point, the first evidence of TGF-β transcript upregulation was noted, consistent with the appearance of immunohistochemical staining for TGF-β in the activated fibroblasts (Westwood et al., 2009). In a separate study, appearance of TGF-β in the plasma of two female dogs given a daily dose of 20 mg/kg AZM551248 was noted after 16 days treatment (Fig. 7). This is broadly consistent with the

### Table 2: Summary of Subcutaneous FD Incidence and Severity During AZM551248 Study

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Day 4</th>
<th>Day 8</th>
<th>Day 11</th>
<th>Day 14</th>
<th>Day 17</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of significant gene expression changes&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/A</td>
<td>85 †, 136 ‡</td>
<td>106 †, 122 ‡</td>
<td>165 †, 340 ‡</td>
<td>1120 †, 1389 ‡</td>
<td>1372 †, 1575 ‡</td>
</tr>
<tr>
<td>Brief summary of histopathological changes</td>
<td>Normal</td>
<td>Normal</td>
<td>Minimal to moderate severity of FD in two of five animals.</td>
<td>Minimal to severe FD severity in all animals.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note.* Direction of transcriptional change is indicated by up and down arrows.  
<sup>a</sup> Signal intensity > 20, fold change > 2x relative to vehicle group with p < 0.05.
upregulation of TGF-β transcripts and the appearance of immunoreactive TGF-β in the subcutis at day 11.

Other observations at the day 11–17 time points, when the subcutaneous fibrotic changes become progressively more severe, suggest the importance of TGF-β as a mediator of these events. Specifically, the genes that are known to be transcriptionally upregulated by TGF-β, and that are observed to be upregulated in this study, are listed in Table 3. Bone morphogenic protein-1 (BMP-1) and integrin αvβ5, which are both upregulated at later time points, are known to be involved in latent TGF-β processing (Asano et al., 2006; Ge and Greenspan, 2006).

Finally, transcripts for the genes encoding a number of collagens, and genes involved in procollagen processing, are significantly upregulated at day 11 onward. In particular, these are collagens 1A1, 1A2, 3A1, 5A1, 5A2, 6A1, and 6A3 and the procollagen processing genes BMP-1, ADAMTS, and the lysyl oxidase precursor LOXL2. Also of note is the upregulation of SerpinH1/HSP47, a specific chaperone for procollagen types I and III (Taguchi et al., 2011), which is upregulated at days 14 and 17.

DISCUSSION

The aim of the studies described here is to associate the pathological stages of FD development in the subcutis with key events.
gene expression changes, thereby elucidating the molecular events associated with AZM551248-induced FD in the dog.

We focused on the early transcript changes to identify the key molecular events induced by AZM551248 treatment. As indicated above, AZM551248 has a relatively broad spectrum of activity, inhibiting a number of MMPs. Interestingly, at day 4, the transcript levels of a number of these MMPs were upregulated in subcutaneous tissues (Fig. 4), although no histopathological changes were evident. This “compensatory” upregulation of gene transcripts, encoding proteins whose abundances or activities are reduced, is a familiar occurrence observed in a variety of biological contexts. For example, inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase enzyme activity in primary human hepatocytes with pravastatin resulted in compensatory increase of HMG-CoA reductase mRNA (Kocarek et al., 2002). Similarly, in animal models of Prader-Willi syndrome, transcript levels of a number of genes encoding pancreatic hormones are upregulated in response to plasma deficiencies in these hormones (Stefan et al., 2011).

Such compensatory regulation, which can occur rapidly as an adaptive response to protein downregulation, is likely to be a consequence of modulation of transcription factor activity. GeneGo pathway mapping highlighted the role of Sp1, RelA, and STAT1 as the key transcription factors in this early gene regulation (Fig. 5). As expected, the pathway included MMP 1 and MMP 2; these proteases are inhibited by AZM551248, and thus, the corresponding transcripts could be expected to be upregulated via a compensatory mechanism.

Consistent with the perturbation of cell adhesion and ECM remodeling, it is noteworthy that the proteases ANPEP, Cathepsin K, and DPP4 are among the relatively few genes significantly regulated at day 4.

In particular, DPP4 (also known as fibroblast activation protein-alpha, FAP-α) provides a potential link with some of the later pathological effects of AZM551248. DPP4 is a prolyl-peptidase (for review, see Juillerat-Jeanneret and Gerber-Lemaire, 2009) and as such ECM proteins and procollagens are likely to be good substrates for this enzyme. In addition to its proteolytic effects, DPP4 activity would also generate free proline within the ECM, which is required by myofibroblasts for collagen synthesis. Thus, DPP4 upregulation, induced by AZM551248, generates a profibrotic mediator, i.e., proline. In support of the profibrotic role for DPP4, inhibitors of DPP4 activity reduce migration of endothelial cells and fibroblasts.

![FIG. 7. Abundance of TGF-β in plasma from AZM551248-treated dogs. Two animals were used in the study, represented by separate lines with open and filled symbols.](image)

![FIG. 8. Schematic summary of key changes across the time course of AZM551248 treatment. Above the line: important histopathological changes, below: gene expression changes.](image)
into collagenous matrices (Ghersi et al., 2006) and antagonize the profibrotic effects of TGF-β in skin fibroblasts (Thielitz et al., 2007).

Another peptidase, the aminopeptidase ANPEP, is also regulated transcriptionally at day 4. ANPEP substrates include ECM proteins (Bank et al., 2006), and ANPEP also modulates TGF-β signaling and is coexpressed with DPP4 in certain hyperproliferative skin disorders (Thielitz et al., 2006).

One consequence of this early peptidase-driven ECM remodeling and fibroblast proliferation/activation is generation of active TGF-β, which can be detected by immunostaining at day 11 (Westwood et al., 2009). This is likely to occur locally via latent pro-TGF-β processing, because transcriptional upregulation of TGF-β is not observed in this study until day 11. BMP-1 and integrin αvβ5, which are known to be involved in latent TGF-β processing, are themselves upregulated by TGF-β, thus creating a positive feedback loop. It is also notable that certain MMPs are involved in TGF-β processing, specifically MMP 2, 3, 9, and 14 (Webster and Crowe, 2006), and transcripts for these genes are upregulated in this study. However, these MMPs would not be active in this context because they are inhibited by AZM551248. Local active TGF-β then triggers a profibrotic cascade, including upregulation of its own synthesis, which drives the development of the observed fibrotic changes as the time course proceeds.

The late stage changes, characterized by extensive collagen deposition, are concomitant with the upregulation of expression of collagens and genes pertinent to procollagen processing (Table 3). In addition to collagens 1A1, 1A2, 3A1, 5A1, 5A2, 6A1, and 6A3, the upregulated procollagen processing genes include the proteinases BMP-1 and ADAMTS and the lysyl oxidase precursor LOXL2, which is involved in collagen cross-linking and assembly (Bignon et al., 2011). Also of note is the upregulation of SerpinH1/HSP47, a specific chaperone for procollagen types I and III, which is upregulated at days 14 and 17. HSP47 is also inducible by TGF-β and is associated with a variety of fibrotic conditions (Taguchi et al., 2011).

In summary, we propose a model to explain FD development following AZM551248 administration, similar to that observed with other nonselective MMPis. This is schematically represented in Figure 8.

The inhibition of key MMPs, specifically MMP 1, 2, 3, 9, and 13, results in compensatory upregulation of MMP transcripts at day 4, driving enhanced transcription factor activity that upregulates factors that destabilize ECM homeostasis, specifically certain peptidases. This results in ECM remodeling, fibroblast proliferation and activation, and ultimately development of overt fibrosis driven by profibrotic factors including TGF-β. Consistent with this model is the observation that inhibitors highly selective for MMP 13 appear to have reduced capacity to induce FD, at least in short-term studies in rat (Baragi et al., 2009; Johnson et al., 2007). In addition to supporting broad-spectrum MMP inhibition as a key feature of compound-induced FD, our data further implicate inhibition of MMP 1, 2, 3, and 9 specifically as being important to FD development. Thus, for the avoidance of FD/MSS, candidate therapeutic MMPis would need to lack inhibitory activity for these proteases.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.
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