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Curcuminoids Inhibit the Angiogenic Response Stimulated by Fibroblast Growth Factor-2, Including Expression of Matrix Metalloproteinase Gelatinase B*

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We have studied mechanisms controlling activation of the gelatinase B gene (matrix metalloproteinase-9) by fibroblast growth factor-2 (FGF-2) during angiogenesis, and the effects of the natural product curcuminoids on this process. Using a transgenic mouse (line 3445) harboring a gelatinase B promoter/lacZ fusion gene, we demonstrate FGF-2 stimulation of reporter gene expression in endothelial cells of invading neocapillaries in the corneal micropocket assay. Using cultured corneal cells, we show that FGF-2 stimulates DNA binding activity of transcription factor AP-1 but not NF-κB and that AP-1 stimulation is inhibited by curcuminoids. We further show that induction of gelatinase B transcriptional promoter activity in response to FGF-2 is dependent on AP-1 but not NF-κB response elements and that promoter activity is also inhibited by curcuminoids. In rabbit corneas, the angiogenic response induced by implantation of an FGF-2 pellet is inhibited by the co-implantation of a curcminoid pellet, and this correlates with inhibition of endogenous gelatinase B expression induced by FGF-2. Angiostatic efficacy in the cornea is also observed when curcuminoids are provided to mice in the diet. Our findings provide evidence that curcuminoids target the FGF-2 angiogenic signaling pathway and inhibit expression of gelatinase B in the angiogenic process.

Angiogenesis, the formation of capillaries derived from a pre-existing vacuature, is central to a wide range of debilitating human pathologies, including solid tumor growth, arthritis, corneal ulceration, and proliferative retinopathies (1, 2). Accumulating evidence implicates members of the matrix metalloproteinase (MMP) family in this process, in particular, gelatinase A (MMP-2) and gelatinase B (MMP-9) (3). These enzymes may contribute to growth of new capillaries in several ways including activation of growth factors that stimulate endothelial cell migration and tube formation and dissolution of endothelial basement membranes at the sprouting capillary tips (4–6). Gelatinase A (MMP-2) is constitutively present in tissues in a latent 72-kDa proenzyme form (7). When required, progelatinase A is proteolytically activated to a 62-kDa form by cleavage of the N terminus by membrane-type MMPs (8); in endothelial cells, this occurs through ligation to the integrin receptor α5β1 (9). In contrast, gelatinase B (92-kDa MMP-9), like most other MMPs, is expressed only upon demand, and regulation occurs at the level of gene transcription (10). Mice made genetically deficient in gelatinase A, although revealing no developmental abnormalities, display impeded angiogenic response to tumor stimulus (11). Targeted inactivation of gelatinase B causes a transient delay in bone development because of defective vascular invasion (12). These gene knockout studies provide the most definitive evidence to date for the importance of the gelatinolytic MMPs in angiogenesis.

Much attention is currently focused on development of synthetic agents that block MMP enzymatic activity as a means to pharmacologically manage diseases involving these enzymes (13). However, considering that most MMPs are expressed only on demand, an alternative approach might target mechanisms for MMP transcriptional activation (10, 14). A highly conserved AP-1 transcription factor DNA-binding site is found in many MMP gene promoters and is thought to be a common thread to their co-ordinate induction in response to diverse stress stimuli (10). AP-1 is activated in response to hypoxia (15), a physiological stimulator of angiogenesis (16). Fibroblast growth factor-2 (FGF-2) is another important angiogenic stimulator (17, 18) that initiates signaling mechanisms ultimately activating AP-1 (19–21). A response element for a second transcription factor activated by stress stimuli, NF-κB, has been found only in the gelatinase B promoter thus far and is one factor determining the unique expression pattern of this gene (10). NF-κB, an oxidative-response transcription factor, also triggers gene expression associated with the angiogenic response (22) by engaging inflammatory cytokine signaling (23). Therefore, targeted inhibition of AP-1 or NF-κB might be a logical step in attempting to modulate the angiogenic response.

Curcuminoids, natural products of the Indian spice turmeric, are potent antioxidant and antiinflammatory agents that have been entered into Phase I clinical trials for chemo-prevention by the National Cancer Institute (24). At least part of their biological activity can be attributed to their capacity to inhibit activation of AP-1 and NF-κB transcription factors. Thus, curcuminoids have been shown to inhibit activation of these transcription factors in response to phorbol myristate acetate (PMA), tumor necrosis factor-α, and hydrogen peroxide (25, 26).

In vascular endothelial cells, curcuminoids reduce the activation of tissue factor gene expression induced by tumor necrosis factor-α, lipopolysaccharide, PMA, and thrombin resulting from the Vision Research Laboratories of New England Eye Center and the Departments of Ophthalmology and Anatomy and Cellular Biology, Tufts University School of Medicine, Boston, Massachusetts 02111.

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§The abbreviations used are: MMP, matrix metalloproteinase; β-gal, β-galactosidase; EMSA, electrophoretic mobility shift assay; FGF-2, fibroblast growth factor-2; PMA, phorbol 12-myristate 13-acetate; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; CAT, chloramphenicol acetyltransferase; ROS, reactive oxygen species.

This paper is available on line at http://www.jbc.org
from the coordinate inhibition of AP-1 DNA binding activity and nuclear translocation of NF-κB (27, 28). In this study, we investigate curcuminoid effects on FGF-2 activation of the gelatinase B transcriptional promoter and on angiogenesis in a corneal micropocket assay. We report that curcuminoids inhibit FGF-2-induced angiogenesis when delivered locally or in the diet in coordination with their down-regulation of transcription factor AP-1 DNA binding activity and of gelatinase B promoter activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatments**—Stromal cells were isolated from rabbit cornea and subcultured as described previously (29). For an experiment, cells were plated, then changed the next day to serum-free medium, and incubated for 24–36 h to reduce basal activity of the transcription factors under investigation. Cells were then pretreated with 20 μM curcuminoids for 30 min, followed by stimulation with either PMA (1 μM) or FGF-2 (50 ng/ml) plus heparin (30 ng/ml). For electrophoretic mobility shift assay (EMSA), treatments were performed for 2–4 h in serum-free medium. For transfection analysis, treatments were performed for 24 h.

**EMSA**—Nuclear lysates were prepared as described previously (30). Protein concentrations of the nuclear extracts were determined using the Bio-Rad reagent, and aliquots of equal protein were frozen at −70 °C. Double-stranded oligonucleotides containing the consensus DNA-binding sites for transcription factors AP-1 (5′-GGGTTAAGGGTTCAGCCGGAA-3′) and NF-κB (5′-AGTTGAGGGAGCTTTCCAGG-3′) served as probes for EMSA (Promega Co., Madison, WI). An oligonucleotide containing a mutated and nonfunctional AP-1-binding site (AP-1*-5′-CGGCTGTTAGGTTCCAGGGG-3′) was employed in competition controls. The oligonucleotides were labeled with [γ-32P]ATP, diluted, and used as probes in EMSA, according to standard methods (30). Antibodies to NF-κB family members p50, p65, and c-Rel were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). For supershift EMSA, 1 μg of one of these antibodies was added to the nuclear lysates, and the radiolabeled probe was incubated for 40 min before electrophoresis. Antibodies to the unrelated transcription factor AP-2 served as a negative control.

**Transfection Experiments**—Rabbit corneal fibroblasts plated in 6-well dishes were transfected with 1 μg of a specific gelatinase B promoter-CAT plasmid, the confection of which was previously described (31). To normalize transfection efficiencies, cultures were cotransfected with 0.5 μg of a cytomegalovirus promoter/β-gal reporter construct (Stratagene, La Jolla, CA). An exception was the experiments involving curcuminoid treatment; these were not normalized to β-gal, because the curcuminoids inhibited cytomegalovirus promoter activity (data not shown). One day after transfection, cell cultures were placed in serum-free medium, and treatments were added as appropriate to the experiment. Cultures were then harvested, and CAT and β-gal activities were assayed from triplicate samples (31).

**Rabbit Corneal Micropocket Angiogenesis Assay**—Prelots of corneal tissues (2 mg, 1.5 mm in diameter) were made using a pellet press (Parr Instrument Co., Moline, IL). Slow release (sucralfate)-FGF-2 pellet was surgically implanted in one cornea in each of ten CD-1 female mice (Charles River Labs, MA) according to the well established procedure of J. Folkman and co-workers (32). One group of five mice harboring the FGF-2 pellets was allowed to feed ad libitum on 1 g curcuminoids/kg chow for a period of 10 days starting on the day of the surgery. The control group of five mice harboring the FGF-2 pellets was allowed to feed on regular rodent chow ad libitum. On days 6 and 10, mice were anesthetized, and their eyes were photographed under a dissecting stereo microscope. In a second experiment, six CD-1 female mice were allowed to feed ad libitum on 2 g curcuminoids/kg chow for 7 days prior to surgical implantation of the 80-ng FGF-2 pellets and continued on the curcuminoid chow for an additional 6 days post-surgery. The control group of six mice was allowed to feed ad libitum on regular chow over the entire period, prior to, and post-surgical implantation of FGF-2. The eyes of the control and curcuminoid-treated mice were photographed on day 6 post-surgery.

In a separate angiogenesis experiment, transgenic mice (line 3445; C57BL/6J background) that contain the DNA sequences between −522 and +19 of the rabbit gelatinase B gene promoter fused transcriptionally to the coding sequences of the bacterial LacZ gene were used. The generation of these mice and the embryonic developmental and wound-induced stimulation of reporter gene expression has been reported (30). Line 3445 transgenic mice were implanted with 80-ng FGF-2 corneal pellets. Developing new blood vessels were followed by examination with a dissecting microscope. By days 3 and 7, gelatinase B expression was barely evident in each cornea. From time to time, paraffin sections were cut and stained for gelatinase B expression. By day 10, gelatinase B expression was evident in the corneal stroma.

**Quantitation of Neovascularization in Mouse Corneas**—We attempted to quantitate corneal blood vessel density by the computer-assisted method of J. Folkman and co-workers (35); however, because CD-1 mice lack a pigmented iris, we could not obtain high contrast photographic images for direct quantitation of blood vessels from photographic prints. Therefore, 35-mm color slide images of neovascularized eyes of each mouse were obtained, and the total number of blood vessels in each cornea was quantified by analyzing traced images of blood vessels. In brief, using a slide projector the slice of neovascularized cornea was projected at a fixed distance to produce an enlarged picture that fit within a piece of 8.5 × 11 inch white paper. Using a 5 × 5 grid, each cell was counted. The area of the enlarged image was traced by hand onto the sheet of paper. The slides were coded to avoid a masked analysis by the investigator, and the tracings were repeated by a second investigator blinded to the study. The traced image of blood vessels of each mouse cornea was scanned using a laser scanner (ScanJet 4C) into a Macintosh computer using the Deskscan and Photoshop software programs in grayscale format at

**Preparation and Provision of Curcuminoid Diet**—Curcuminoids purchased from Sigma, sold under the name curcumin, contain approximately 77% curcumin, 17% demethoxycurcumin, and 3% bisdemethoxycurcumin (34). The curcuminoid-supplemented diet was prepared by mixing 1 g (experiment 1) or 2 g (experiment 2) of curcuminoids/kg of standard rodent chow. In brief, the rodent chow was pulverized into a powder and thoroughly blended with the curcuminoid mixture. The chow mix was wetted with sterilized distilled water, reconstituted into nugget size pieces, and immediately frozen at −20 °C. Adequate amounts of the curcuminoid-supplemented chow were brought to room temperature and provided fresh each day to the mice. Any remaining chow that was not consumed was discarded and replaced with a fresh batch the next day.
8-bits/pixel resolution. The imported images from Photoshop were directly transferred without any alterations into the NIH Image analysis software, and the sum of all vessels in a cornea were quantified as pixels. The data in the control and curcuminoid-treated groups were decoded, and results of the analysis performed by the two researchers were averaged. Student's t test was applied to the data to obtain significance values.

RESULTS

Gelatinase B Is a Downstream Target in the FGF-2 Angiogenic Gene Expression Program—We have recently reported our development of a transgenic mouse line (3445) harboring a lacZ reporter gene driven by the −522 to +19 of the rabbit gelatinase B transcriptional promoter (30). We showed that this promoter fragment is sufficient to drive embryonic and injury-induced reporter gene expression in a manner that mimics the pattern of the endogenous gelatinase B gene. In this study, we used line 3445 mice to investigate activation of the gelatinase B promoter to an FGF-2 angiogenic stimulus using the corneal micropocket assay. Implantation of slow release pellets containing 80 ng of FGF-2 into the mouse cornea stimulated growth of corneal capillaries from the limbal vasculature located at the transitional zone between the cornea and sclera. These new blood vessels were abundant by 7 days post-surgery when observed under a dissecting microscope (Fig. 1A, small arrows). The neovascularized eyes were analyzed by whole mount histochemical staining for reporter gene expression. β-Gal activity was observed around the FGF-2 pellets and in a punctate pattern paralleling the capillary network growing toward the pellets (Fig. 1B, small arrows). Finally, staining was seen within the area of the pre-existing limbal vessels (Fig. 1B, large arrow); however, dissection of the tissue revealed that most of this staining occurred in underlying structures in the angle of the anterior chamber (such as the ciliary body), which also stained in eyes without implanted pellets (data not shown). In thin tissue sections of the X-gal-stained neovascularized portions of the cornea, β-gal activity was revealed to occur in cells composing the vessel lumen and cells that had accumulated around the new capillaries (Fig. 1C). A time course study revealed that gelatinase B promoter activity was transient within the vascularizing tissues; migratory endothelial cells stained by day 2 and outlined newly formed blood vessels till day 7, becoming sporadic after this time point (data not shown). The fellow eye of the same mouse that lacked FGF-2 pellet or eyes of transgenic mouse line 3445 implanted with pellets lacking growth factor did not develop corneal blood vessels and failed to show corneal β-gal staining at 7 days post-surgery (data not shown). Together, these results indicate that activity of the gelatinase B transcriptional promoter is spatially and temporally regulated during the process of angiogenesis that occurs in response to FGF-2 stimulus.

Role of AP-1 DNA-binding Elements in Response of the Gelatinase B Promoter to FGF-2 and Inhibition by Curcuminoids—the effect of curcuminoids on the FGF-2-mediated activation of two transcription factors known to contribute to gelatinase B promoter activity, AP-1 or NF-κB, was investigated by EMSA. As a positive control, we confirmed the known activity of curcuminoids against transcription factors activated in response to PMA (25, 26). Cells were serum-starved for 24–36 h to reduce transcription factor activities to basal levels prior to initiating an experiment.

When radiolabeled consensus oligonucleotide probes for AP-1 or NF-κB were incubated with nuclear lysates prepared from untreated serum-free cells, a single major DNA-protein complex formed in each case (arrowheads), as revealed by the presence of a band with retarded electrophoretic mobility in comparison to the free probe (Fig. 2, A and B, SF lanes). The specificity of the complex that formed on the radiolabeled AP-1 oligo was revealed by its abrogation when competed with a 50-fold excess of cold AP-1 oligo, but not by a 50-fold excess of the mutant AP-1 oligo (AP-1*) containing two nucleotide changes in the consensus DNA sequence (Fig. 2, A and B, NSB lanes). The specificity of the major complex that formed on the radiolabeled NF-κB oligo was similarly revealed by competition with a 50-fold excess of cold probe (Fig. 2A, 1:50 oligo lanes), but not by a nonspecific binding control, cold AP-1 probe (Fig. 2A, NSB lane). Furthermore, antibodies to the NF-κB subunits, p50 and p65, each supershifted a subcomponent of the major complex, but antibodies either to NF-κB subunit c-Rel or to the unrelated transcription factor AP-2 had no effect (Fig. 2A). The nonspecific nature of the faster migrating minor complex (Fig. 2A, asterisk) in NF-κB EMSA is indicated by its unresponsiveness to competition or antibody supershift.

PMA treatment of cells stimulated AP-1 and NF-κB DNA binding activities as previously reported (25, 26) (Fig. 2A). In contrast, FGF-2 treatment stimulated AP-1 DNA binding activity, but NF-κB DNA binding activity was not altered (Fig. 2B). Curcuminoid treatment of otherwise untreated cells for 30 min did not alter the basal DNA binding activity to the two probes under examination (Fig. 2, A and B, compare SF and...
FIG. 2. Differential inhibition of transcription factors by curcuminoids in corneal fibroblasts stimulated by FGF-2 and PMA. Serum-starved corneal fibroblasts left untreated in serum-free medium (SF) or treated with 20 μM curcuminoids (Cur) were treated 30 min later with 1 μM PMA and incubated for 4 h (A) or 50 ng/ml FGF-2 and incubated for 2 h (B). Nuclear extracts prepared from these cells were incubated with radiolabeled AP-1 or NF-κB consensus oligonucleotides and analyzed by EMSA. For the AP-1 analysis, competition was performed with a 50-fold excess of cold AP-1 probe or a mutant AP-1 oligonucleotide (AP-1*). For the NF-κB analysis, competition was performed with a 50-fold excess of cold nonspecific probe AP-1 or with a 10-fold (1:10) or 50-fold (1:50) excess of cold NF-κB probe. Supershift analysis was performed with antibodies to the NF-κB proteins p50, p65, and c-Rel. An antibody to transcription factor AP-2 formed with a 50-fold excess of cold nonspecific probe AP-1 (NSB) or with a 50 ng/ml FGF-2 and 1:50 electrophoretic mobility shift assay (EMSA) antibody. The antibody to transcription factor AP-2 served as a negative control. The position of supershifted complexes is indicated by the long arrows. The position of the specific oligonucleotide binding complexes identified by these controls are indicated by the arrowheads. The nonspecific protein-DNA complex that binds the NF-κB probe is denoted by an asterisk.

Cur lanes. However, curcuminoid treatment inhibited the PMA stimulation of DNA binding activities for AP-1 and NF-κB, as previously reported (Fig. 2A). The new finding of this study was that curcuminoid treatment had a similar effect on transcription factor activation by FGF-2. Thus, activation of AP-1 DNA binding activity stimulated by FGF-2 was inhibited, but basal DNA binding activity was unaffected (Fig. 2B). These findings indicate that curcuminoids distinguish between AP-1-dependent promoter activity and basal DNA binding activity. Furthermore, although FGF-2 activates different signaling pathways than PMA in corneal stromal cells, curcuminoids are as effective in blocking the FGF-2 pathway as they are in blocking the PMA-stimulated signaling pathway.

To determine the role of NF-κB and AP-1 response elements for gelatinase B promoter activity, we performed transfection experiments with a series of gelatinase B promoter-reporter gene fusion constructs (Fig. 3A). The first construct, −519 CAT (encompassing promoter sequences between −519 and −19) lacks a functional NF-κB site since the 5’ truncation removes four of the ten consensus nucleotides required for NF-κB-mediated transcription (31). However, this construct retains the distal and proximal AP-1 site (30, 31). Treatment of Pr 21-transfected corneal fibroblasts with 50 ng/ml FGF-2 induced reporter gene expression by over 2-fold. In contrast, a cut-back construct lacking the distal AP-1 site (−423 CAT) failed to respond to FGF-2 stimulation, although substantial basal levels of promoter activity were observed. Mutagenesis of the proximal AP-1 DNA-binding site in −519 CAT (−519(AP1-Prox) CAT) (31) also abrogated FGF-2-stimulated reporter gene expression and inhibited basal promoter activity as well. This indicates that the proximal AP-1 site is necessary for gelatinase B promoter activity and is required along with the distal AP-1 element to confer transcriptional response to FGF-2.

We next addressed the question of whether gelatinase B promoter activation induced by FGF-2 could be inhibited by curcuminoids (Fig. 3B). Addition of as little as 50 nM curcuminoids to −519 CAT-transfected cultures resulted in complete abrogation of FGF-2-stimulated promoter activity, bringing reporter gene expression back down to the basal level. In contrast, treatment of −519 CAT-transfected cultures with 50 nM curcuminoids did not alter basal levels of promoter activity. Thus, curcuminoids distinguish between AP-1-dependent promoter activation by FGF-2 and basal activity of the promoter in agreement by our EMSA results.

Curcuminoids Reduce the Angioproliferative Response in Vivo and Inhibit the Appearance of Specific Gelatinase Forms in the Cornea—To begin to examine the effects of curcuminoids on angiogenesis stimulated in response to FGF-2, we again utilized the corneal micropocket assay. This time, however, we used rabbits because of the larger corneal size and thus availability of more tissue for biochemical analysis. Three groups of four rabbits each were analyzed. In group one, each rabbit was implanted with a single 80-ng FGF-2 pellet. In group two, each rabbit was implanted with a 2-mg curcuminoid pellet placed in a micropocket adjacent to the FGF-2 pellet. In group three, each rabbit was implanted with a single 2-mg curcuminoid pellet. The rabbit corneas are considerably larger than mouse corneas; therefore, the 80-ng FGF-2 pellets were not as effective in stimulating angiogenesis as in the experiment described above. Nevertheless, rabbits implanted with FGF-2 pellets had developed limbal vessel dilatation in response to the angiogenic stimulus of the growth factor when examined 7 days post-surgery. By the end of 12 days, the group of rabbits treated with only FGF-2 continued to show limbal vessel dilatation, and corneal angiogenesis was most noticeable at the limbus (Fig. 4B), where as rabbits harboring FGF-2 and curcuminoid pellets showed no limbal vessel dilatation and angiogenesis (Fig. 4C). In fact, these corneas looked the same as they did prior to implantation of FGF-2 pellets. Because vessel growth was not optimally stimulated by FGF-2 in this model, we did not attempt to quantify angiogenesis. However, we observed that all the rabbit corneas implanted with FGF-2 and curcuminoid pellets demonstrated attenuated angiogenic response that was consistent within this group. These data also suggested that curcuminoids produced a vasosuppressive effect on FGF-2-induced vessel dilatation. The rabbits treated with curcuminoid pellets alone (Fig. 4A) showed no signs of eye irritation, inflammation, or corneal opacity, suggesting that curcuminoids as implantable drugs were relatively safe to the anterior ocular surface.

We next investigated the effects of curcuminoid pellet implantation on gelatinase B expression in the rabbit corneas. Analysis of extracts by zymography from central regions of the four corneas implanted with only curcuminoid pellets revealed a gelatinolytic protein that migrated at 65 kDa (Fig. 5). We identify this protein as pro-gelatinase A based on its co-migra-
tion with rabbit pro-gelatinase A produced by cultured corneal stromal cells. The presence of progelatinase A is consistent with our previous studies that have identified this MMP as a normal component of the corneal stroma (29, 36, 37). A minor gelatinolytic protein was also present at 62 kDa; this size is appropriate for the proteolytically activated form of gelatinase A. Extracts from FGF-2-pellet implanted corneas also contained the 65-kDa and minor 62-kDa gelatinase A forms. However, a new gelatinolytic activity migrating at 92 kDa was also apparent. This new activity was clearly present in all four of the FGF-2-treated corneas. Based on co-migration with the rabbit progelatinase B standard, we identify this enzyme as progelatinase B. Addition of curcuminoid pellets did not alter wound healing of rabbit corneas harboring FGF-2 pellets to any extent apparent by gross observation. However, FGF-2-stimulated appearance of gelatinase B was completely inhibited in all four curcuminoid-implanted corneas. These results indicate that curcuminoids inhibit FGF-2-stimulated expression of gelatinase B, in parallel with inhibition of FGF-2 stimulation of the angiogenic response.

Dietary Curcuminoids Inhibit Corneal Angiogenesis in Mice—We returned to the mouse model for a more quantitative analysis of the effects of curcuminoids on corneal angiogenesis. In this study, we also chose to examine the efficacy of an oral route for curcuminoid delivery, through dietary supplementation. After implantation of a single 80-ng slow release FGF-2 pellet in one eye of each mouse, one group of mice was placed on the curcuminoid-supplemented (1 g curcuminoids/kg chow) diet, whereas the control group was fed regular rodent chow. Mice were allowed to feed and drink water ad libitum. The implanted corneas of mice were photographed on days 6 and 10 after FGF-2 pellet implantation to visualize the development of new blood vessels. Angiogenesis was quantified in each cornea by measurement of total sum of blood vessel number and length. Mice fed a diet containing curcuminoids were found to have fewer and less tortuous blood vessels compared with the

FIG. 3. AP-1 mediates FGF-2-stimulated gelatinase B promoter activity and inhibitory response of curcuminoids on gelatinase B promoter activation. Rabbit corneal fibroblasts were transfected with Gel B/CAT promoter constructs and assayed for CAT activity after treatments with FGF-2 and/or curcuminoids. A diagram of the full-length -519 CAT construct is shown, with the two AP-1 response elements indicated. A, transfected cells were incubated in triplicate with or without 50 ng FGF-2 for 24 h. The construct -519 CAT contains two AP-1 response elements. The distal AP-1 element is deleted in -423 CAT. The proximal AP-1 element mutated in the -519 (AP1-Prox) CAT construct (31). B, cells were transfected with -519 CAT and then treated for 24 h with 50 ng curcuminoids, 50 µg of FGF-2, or a combination of the two agents.

FIG. 4. Localized delivery of curcuminoids inhibits the angioproliferative response to FGF-2 stimulation in rabbit corneas. Photograph of rabbit eyes showing the cornea-scleral region of neovascularized corneas 12 days post-surgery. The slow release 80-ng FGF-2 pellet in B and C are indicated by the asterisks, and the 2-mg curcuminoid pellet in A and C appear as yellow circular discs.

FIG. 5. Localized delivery of curcuminoids reduces FGF-2-induced expression of gelatinase B in neovascularized corneal tissues. A circular region of neovascularized tissue 5 mm in diameter encompassing the FGF-2 pellets was isolated from the corneas of each of the four rabbits in the three different treatment groups. Equal amounts of SDS-extracted proteins from each tissue specimen were subjected to gelatin zymography. The presence of specific gelatinases was revealed by the clear bands in the stained gelatin background. Identification of enzymes was made by comparison of migration position to a rabbit corneal cell gelatinase standard (Std): gelatinase B at 92 kDa (GelB), gelatinase A proenzyme at 65 kDa (pGelA), and a proteolytically activated form of gelatinase A at 62 kDa (GelA). The arrow indicates the glycosylated form of gelatinase B, and the arrowhead indicates the unglycosylated form.
Angiostatic Activity of Curcuminoids

A chow containing 1 g curcuminoids/kg chow. Representative photographic images of mouse corneas taken at day 6 (A and B) and day 10 (C and D) reveal the presence of corneal blood vessels. A and C are representatives from the control group, whereas B and D are from the curcuminoid-fed group. The asterisk indicates the position of the FGF-2 pellet.

Control group on day 6 post-surgery (Fig. 6, A and B). In addition, the limbal and corneal blood vessels of the curcuminoid-fed group were not as dilated as the control group. An even greater difference between the experimental and control groups in these parameters was observed in mice fed curcuminoids for an additional 4 days (Fig. 6, C and D). Quantitation of the angiostatic effect of curcuminoids revealed a greater than 60% inhibition of new blood vessel growth (Table I), which was significant (p < 0.007).

We also evaluated the possibility that prefeeding mice a diet containing curcuminoids prior to FGF-2 stimulation might have therapeutic effects. In this experiment, the test group of mice was fed a diet of curcuminoids (2 g/kg chow) for 1 week prior to surgical implantation of FGF-2 pellets and continued on this chow for 6 days post-surgery; the control group was maintained on normal chow throughout the entire period. By as early as day 6 post-surgery, new blood vessel growth was inhibited by greater than 50% (Table I) in the curcuminoid-treated mice compared with the control group, which was also significant (p < 0.0002). Together, these findings reveal that curcuminoids harbor potent angiostatic activity also when provided in the diet.

DISCUSSION

We have employed a gelatinase B promoter/lacZ transgenic mouse (line 3445) and a corneal micropocket angiogenesis model to demonstrate that gelatinase B is a downstream target in the FGF-2-regulated angiogenic pathway. Using cultured corneal cells, we show that FGF-2 stimulates DNA binding activity of transcription factor AP-1 but not NF-κB and that stimulation of AP-1 is inhibited by curcuminoids. We further show that induction of gelatinase B promoter activity in response to FGF-2 is dependent on AP-1, but not NF-κB, response elements and that promoter activity is also inhibited by curcuminoids. When curcuminoids were delivered locally to the cornea via an implantable pellet, the angiogenic response to FGF-2 was inhibited, including induction of endogenous gelatinase B expression. This finding is in keeping with previous studies demonstrating targeting of gelatinase B expression by curcuminoids (45, 46). The angiostatic activity of curcuminoids on FGF-2-stimulated angiogenesis was further demonstrated by dietary provision to mice. To our knowledge, this is the first study showing the efficacy of curcuminoids as implantable drugs for inhibition of angiogenesis locally or as orally active drugs to inhibit angiogenesis systemically.

Accumulating evidence implicates both of the gelatinolytic matrix metalloproteinases, gelatinase A and gelatinase B, in the process of angiogenesis. Although these two enzymes have very similar specificities for extracellular components of the basement membrane, their activities are regulated quite differently. Thus, gelatinase A is constitutively present in tissues, primarily in the proenzyme form, and is activated upon demand (36). In contrast, gelatinase B proenzyme expression is transcriptionally regulated. Here, we utilized line 3445 mice to study activation of the gelatinase B promoter during angiogenesis in situ. We previously documented the validity of this mouse model to study the regulation of gelatinase B promoter activity in tissue remodeling events of development and wound healing (30). In this study, we demonstrate that the gelatinase B promoter is spatially and temporally regulated in response to FGF-2 stimulation in a manner that supports the requirement for focal expression of gelatinase B in angiogenesis. Thus, cells of invading corneal capillaries in line 3445 transgenic mice displayed discrete regions of promoter activity, in a manner similar to the localized activity observed in basal cells of the actively migrating epithelial sheet in wound healing skin (30).

Response elements for transcription factor AP-1 are essential for basal activity of the gelatinase B promoter and are required for promoter stimulation by all agents and conditions tested thus far (10, 31, 39, 40). Studies on FGF-2 signaling have identified AP-1 as a final effector of gene expression activated by this pathway (38). The EMSA performed in this study demonstrating activation of AP-1 DNA binding activity by FGF-2 confirmed this report. In contrast, transcription factor NF-κB was not activated by FGF-2 in our cultured stromal cell model. Our transfection experiments corroborated the EMSA findings, revealing that the FGF-2-stimulated transcriptional response of the gelatinase B gene promoter requires AP-1 but not NF-κB response elements. These findings agree with a recent report that activation of NF-κB occurs only on co-stimulation of FGF-2 with inflammatory cytokines such as interleukin-1α and tumor necrosis factor-α (41). Furthermore, lack of requirement for NF-κB is consistent with FGF-2-stimulated angiogenesis in vitro; Stoltz et al. (42) have shown that NF-κB is not activated in endothelial cells when stimulated by FGF-2 to form capillary tubes in Matrigel. Together these findings suggest that AP-1 would offer the best target to interfere with FGF-2 signaling to abrogate angiogenesis and expression of gelatinase B.

Table I

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<th>Treatment schedule</th>
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<td>11693 (2288)</td>
</tr>
<tr>
<td>Pre-treatment for 7 days plus post-treatment for 6 days</td>
<td>10774 (3504)</td>
</tr>
</tbody>
</table>

Effect of dietary curcuminoids on corneal angiogenesis

Values in parentheses are standard deviations.
Curcuminoids exhibit both antioxidant and anti-inflammatory activities. These activities have been ascribed to their ability to scavenge active oxygen and nitrogen species and interfere with lipid peroxidation (reviewed in Ref. 24). Owing to their potent inhibitory activity on arachidonic acid release and metabolism both cyclooxygenase and lipooxygenase pathways are inhibited by these natural products (43). Anticancer activity of curcuminoids has been associated with inhibition of c- myc and c-jun, and c-fos oncogene expression, and signal transduction studies have revealed that this inhibition occurs via inhibition of c-Jun N-terminal kinase activation (44).

Although curcuminoids are known to inhibit AP-1 and NF-κB activation in response to a variety of stimuli (25, 26), we are not aware of previous studies examining their inhibitory effects on FGF-2-stimulated activation. Interestingly, the inhibitory activity of curcuminoids on AP-1 was found to be stimulus-dependent; constitutive DNA binding activities were unaffected as demonstrated in EMSA. In addition, we demonstrate that curcuminoids reduce FGF-2-induced but not basal transcriptional activity of the gelatinase B promoter. These results suggest that curcuminoids act to block some step in the FGF-2 signaling pathway upstream of DNA-binding that becomes engaged upon growth factor stimulation. It is now well known that curcuminoids inhibit production of reactive oxygen species (ROS) that act as intermediates in many signal transduction pathways (24). ROS are a likely target in our case because it has been reported that FGF-2 induces AP-1 activation via ROS produced through NADPH oxidase (38).

Turmeric and its ethanolic extracts (curcuminoids) have been documented as antiinflammatory and antioxidiant agents in treatment of tumors, arthritis, and wound healing disorders, both through oral intake and topical application (50, 51). In fact, dietary curcuminoids have been shown to improve the metabolic status in diabetic condition in rats (52) and demonstrated antarcinogenic effects in several preclinical tests (24, 53, 54). In this study, we show that dietary supplementation with curcuminoids has significant angiostatic effects in mice. However, to demonstrate efficacy the timing and duration of treatment was an important factor. We postulate that curcuminoids acting as antioxidants may exhibit antiangiogenic activities by stimulating redox-regulatory defense systems reviewed in Ref. 55. Given the finding that FGF-2 induces ROS production (38), we speculate that predosing mice with dietary curcuminoids would impinge on this redox pathway and interfere with the ability of FGF-2 to stimulate AP-1. The identification of the target of curcuminoids should throw more light on the molecular mechanisms of its inhibitory activity on FGF-2 signaling.

It is of interest that quite a few biologically active natural products derived from ethnic foods are being explored for the treatment of cancer, arthritis, and wound healing disorders. A recent study has revealed that vascular endothelial growth factor-induced corneal angiogenesis is inhibited by providing green tea extract (containing epigallocatechin-3-gallate) in drinking water to mice (56). The curcuminoids, which are the major active ingredients of the spice turmeric, are considered relatively safe for human consumption by the joint Food and Agriculture Organization of the United Nations/World Health Organization committee pending preclinical toxicity studies (24). This provides an unique opportunity to test the inhibitory activities of curcuminoids in other angioenic models as well. We propose the local delivery and/or systemic administration of curcuminoids to control fibrovascular proliferative diseases where long term therapy may be necessitated.

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