Angiopoietin-like Protein 2 Accelerates Carcinogenesis by Activating Chronic Inflammation and Oxidative Stress

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Abstract

Chronic inflammation has received much attention as a risk factor for carcinogenesis. We recently reported that Angiopoietin-like protein 2 (Angptl2) acts as a chronic inflammatory mediator in several pathologic settings (3–6), and demonstrated that increased Angptl2 expression in skin tissues promotes inflammation and accelerates carcinogenesis in a chemically induced squamous cell carcinoma (SCC) of the skin mouse model. In particular, we demonstrated that Angptl2-induced inflammation enhanced susceptibility of skin tissues to "preneoplastic change" and "malignant conversion" in SCC development; however, mechanisms underlying this activity remain unclear. Using this model, we now report that transgenic mice overexpressing Angptl2 in skin epithelial cells (K14-Angptl2 Tg mice) show enhanced oxidative stress in these tissues. Conversely, in the context of this model, Angptl2 knockout (KO) mice show significantly decreased oxidative stress in skin tissue as well as a lower incidence of SCC compared with wild-type mice. In the chemically induced SCC model, treatment of K14-Angptl2 Tg mice with the antioxidant N-acetyl cysteine (NAC) significantly reduced oxidative stress in skin tissue and the frequency of SCC development. Interestingly, K14-Angptl2 Tg mice in the model also showed significantly decreased expression of mRNA encoding the DNA mismatch repair enzyme Msh2 compared with wild-type mice and increased methylation of the Msh2 promoter in skin tissues. Msh2 expression in skin tissues of Tg mice was significantly increased by NAC treatment, as was Msh2 promoter demethylation. Overall, this study strongly suggests that the inflammatory mediator Angptl2 accelerates chemically induced carcinogenesis through increased oxidative stress and decreased Msh2 expression in skin tissue.

Implications: Angptl2-induced inflammation increases susceptibility to microenvironmental changes, allowing increased oxidative stress and decreased Msh2 expression; therefore, Angptl2 might be a target to develop new strategies to antagonize these activities in premalignant tissue.

Introduction

The connection between cancer and inflammation was first made in the 19th century (1). Since then, epidemiologic studies have confirmed the idea that chronic inflammation underlies many cancers (2). Recently, we reported that Angiopoietin-like protein 2 (Angptl2) acts as a chronic inflammatory mediator in several pathologic settings (3–6), and demonstrated that increased Angptl2 expression in skin tissues promotes inflammation and accelerates carcinogenesis in a chemically induced squamous cell carcinoma (SCC) of the skin mouse model by increasing susceptibility to "preneoplastic change" and "malignant conversion" (7). In normal cells, initiating oncogenic mutation is considered essential to promote a "preneoplastic change," which, if unrepaired, is then followed by proliferation and acquisition of cellular survival capacity, allowing accumulation of additional mutations (8–10). Potentially oncogenic mutations can be antagonized by DNA repair mechanisms, some mediated by members of the mismatch repair (MMR) family, which corrects base mispairings or repairs larger insertion/deletion loops (IDL; refs 11–14). In the MMR system, three homologs of the bacterial MutS protein—Msh2, Msh3, and Msh6—form heterodimers that recognize DNA damage (15). Loss-of-function mutations or epigenetic silencing of MMR genes increase genomic microsatellite instability (MSI), increasing the rate of DNA replication errors (16). Genetic and biochemical studies indicate that Msh2 is a critical component of all MMR complexes and, consequently, Msh2-null mutants are predicted to completely lack...
MMR response (17, 18). Significantly, loss or mutation of mammalian Msh2 increases the probability of developing certain types of tumors (19).

In this study, we report that K14-Angptl2 Tg mice, which show accelerated skin tissue inflammation and greater frequency of chemically induced SCC, showed enhanced oxidative stress in skin tissues and decreased expression of Msh2, likely due to increased methylation of the Msh2 promoter. Interestingly, treatment of these mice with the antioxidant N-acetyl cysteine (NAC) significantly reduced oxidative stress in skin tissue and SCC frequency. Furthermore, Msh2 mRNA expression in skin tissue of Tg mice significantly increased following NAC treatment in parallel with demethylation of the Msh2 promoter region. Overall, these findings suggest that Angptl2-induced inflammation increases susceptibility to microenvironmental changes allowing accumulation of oncogenic DNA mutations.

Materials and Methods

Mice

Male mice were used for the experiments. Mice were housed in a temperature-controlled room with a 12-hour light/dark cycle. Food and water were available ad libitum unless otherwise noted. Mice were fed a normal diet (CE-2;CLEA, Japan). For some experiments, NAC (40 mmol/L) was administered in drinking water from 7 weeks after birth until the end of the experiments. For chemical carcinogenesis assays, K14-Angptl2 Tg (3) and Angptl2 KO (3) were backcrossed to the FVB/N strain for more than 10 generations. During the course of the experiment, we observed no significant difference in body weight between K14-Angptl2 Tg and wild-type littermate mice or between Angptl2 KO and wild-type littermates. In addition, we observed no significant difference in body weight between these groups, with or without NAC treatment. All experiments were performed according to the guidelines of the Institutional Animal Committee of Kumamoto University.

Chemically induced skin carcinogenesis

We performed chemical skin carcinogenesis experiments using K14-Angptl2 Tg mice, Angptl2 KO mice, and respective wild-type littermates as controls, as described previously (20, 21). Briefly, 50 µg of the chemical initiator mutagen 7,12-dimethylbenzanthracene (DMBA; Sigma) was applied topically to the shaved skin on the back of 8-week-old male mice, followed by weekly topical application of 5 µg of the tumor promoter phorbol ester 12-O-tetradecanoylphorbol 13-acetate (PMA; Sigma) over 20 weeks. Raised lesions of a minimum diameter of 1 mm present for at least 1 week were scored as papillomas. Large papillomas were defined as papillomas of 3 mm or more in diameter. Mice were killed after 45 weeks or at 8 weeks after the first diagnosis of SCC. SCC evaluation was carried out clinically and histologically by two independent dermatologists using specified standards (20–22). Clinically, SCCs are significantly larger and grow more rapidly than papillomas. Histologically, papillomas show loss of polarity and anaplasia of the nuclei localized to the epidermis. However, SCCs are invasive carcinomas of the surface epidermis. SCCs proliferate downward into the dermis. The ratio of malignant conversion was calculated for each group of mice as the total number of SCC divided by the number of large papillomas and expressed as a percentage. The two-sided unpaired Student t-test was used to analyze differences in the number of tumors per mice with or without NAC treatment.

Immunoblot analysis

Skin tissues were homogenized in lysis buffer (10 mmol/L NaF, 1 mmol/L Na3VO4, 1 mmol/L EDTA, 300 mmol/L NaCl, 50 mmol/L Tris–HCl, pH 7.5, and 1% Triton X-100). Extracts derived from supernatants were subjected to SDS–PAGE electrophoresis, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using an ECL kit (Amersham) according to the manufacturer’s instructions. Antibodies against 4-hydroxy-2-nonenal (4-HNE; Santa Cruz Biotechnology), Hsc70 (B-6; R&D Systems), Rad51, Brca2, Atm, Atr, Ku70, Ku80, and Msh2 (Cell Signaling Technology), and Angptl2 (3) were used.

Immunohistochemical staining

Skin tissues were fixed by perfusion with 4% paraformaldehyde in PBS (pH 7.4), washed in PBS for 15 minutes, dehydrated through a graded series of ethanol and xylene, and embedded in a single paraffin block. Sections (5 µm) were cut, air-dried, deparaffinized, and pretreated with 5 mmol/L periodic acid for 10 minutes at room temperature to inhibit endogenous peroxidase. Specimens were incubated for 1 hour with 50- to 100-fold diluted polyclonal antibody against Angptl2 (3) or Msh2 (Cell Signaling Technology), and then washed 3 times with PBS for 5 minutes. Sections were incubated with biotinylated anti-mouse IgG or anti-rabbit IgG (1:200 dilution; Vector Laboratories). Immunostaining was performed using the peroxidase-labeled avidin–biotin–complex method (1:100 dilution; DAKO). Sections were counterstained with hematoxylin.

DNA extraction and bisulfate modification

DNA was isolated from skin tissues using the SDS/proteinase K method. DNA concentration was determined spectrophotometrically at 260 nm. DNA bisulfate treatment was used to convert unmethylated CpG sites to UpG without modifying methylated sites, thus allowing them to be distinguished by restriction digestion using the Combined Bisulfite Restriction Analysis (COBRA) assay (17). Bisulfite treatment of genomic DNA was performed using the MethylEasy Xceed Rapid DNA Bisulfite Modification Kit (Human Genetic Signatures) according to the manufacturer’s instructions. CpG-methylated NIH 3T3 mouse genomic DNA (New England Biolabs) served as a positive control and underwent bisulfite modification as described earlier. NIH 3T3 mouse genomic DNA (New England Biolabs) served as a negative control. The proportion of methylated versus unmethylated product (digested vs. undigested) was quantified by densitometry (23). Densitometric analysis was performed using Multi Gauge software (Fujifilm).
PCR and restriction digestion

Msh2 primers were specially designed for the COBRA assay as described previously (17). Sequences used were 5'-TGGCGGTGTAGTTTAAGGAGA-3' (forward) and 5'-TCTTAAACCTCCGCAACC-3' (reverse), generating a 195-bp amplification product (17). PCR amplifications were performed using TaKaRa EpiTaq HS (for bisulfite-treated DNA; Takara Bio) according to the manufacturer’s instructions. After amplification, PCR products were digested with MboI (Promega), which cuts DNA harboring a d(GATC) target site only if the site is retained after bisulfite-mediated deamination. Samples were separated on 2% agarose gels, which were then stained with ethidium bromide. The proportion of methylated versus unmethylated product (digested vs. undigested) was quantified by densitometry (17, 23) using MultiGauge version 3.1 software (Fuji Film).

Calculation of survival data

A Kaplan–Meier log-rank test was applied to analyze mouse survival data using JMP7 software (SAS Institute). A P value of less than 0.05 was considered significant.

Real-time quantitative RT-PCR

For mouse skin tissues, total RNA was isolated using the TRIzol reagent (Invitrogen). DNase-treated RNA was reverse transcribed with a PrimeScript RT reagent Kit (Takara Bio). PCR products were analyzed with a Thermal Cycler Dice Real Time system (Takara Bio), and relative transcript abundance was normalized to that of 18S mRNA. PCR oligonucleotides were as follows: mouse Msh2: forward, 5'-GCCGAGATGCCATTGTTAAAG-3', reverse, 5'-AAC-GGTTGCTCGTGTGTTGAC-3'; mouse 18S: forward, 5'-TTCGCAACAACCTAGCTGACAC-3', reverse, 5'-CC-ACTTCTCACAAGTGGGCTG-3'; mouse IL-1β: forward, 5'-TCCAGGATGAGGACATGAGCAC-3', reverse, 5'-GAACGTCACACACCAGGTTA-3'; mouse IL-6: forward, 5'-CCACTTCTACAATTGCCAGAAGGCTA-3', reverse, 5'-GCAAGTGCTGTTTCTACATC-3'. Results were analyzed using the two-tailed Student t-test with Excel software (Microsoft). A P value of less than 0.05 was considered significant.

Clinical characterization of patients

For ANGPTL2 and MSH2 immunostaining, we obtained normal abdominal skin tissues, as well as tissues derived by curative resection from patients with actinic keratosis and squamous cell carcinoma on the face (at Kumamoto University). Diagnostic evaluation of human samples was carried out by two independent dermatologists clinically and histologically. All studies were approved by the Ethics Committee of Kumamoto University. Written informed consent was obtained from each subject.

Results

Increased oxidative stress parallels Angptl2 expression in mouse skin tissues

Initially, we confirmed our previous findings regarding differences in susceptibility to chemically induced carcino-
tissues (data not shown). Therefore, for further study we analyzed skin tissues from the mid-dorsal portion of the back of animals at 7 weeks after beginning chemical application.

In animal tissues, 4-hydroxy-2-nonenal (4-HNE) is a lipid peroxidation product whose formation is closely related to oxidative stress (24). Because of its electrophilic nature, 4-HNE reacts with nucleophilic amino acid residues in proteins to form HNE-adducted proteins. For our studies, we used an HNE-antibody that recognizes HNE-adducted histidine moieties in proteins (HNE-modified proteins) to monitor both the occurrence and extent of oxidative stress in skin tissues (Supplementary Fig. S1). Immunoblot analysis of skin tissues at the 7-week time point using the anti-4-HNE antibody indicated that HNE-modified protein level, in tissues from K14-Angptl2 Tg mice, significantly increased relative to conditions seen in wild-type mice (Fig. 2A and B). Conversely, levels of HNE-modified proteins in skin tissues of Angptl2 KO mice were significantly decreased compared with wild-type controls (Fig. 2C and D). In addition, we found that HNE-modified protein levels were positively correlated with the Angptl2 levels (Fig. 2A and C). These findings suggest that Angptl2 increases oxidative stress.

Antioxidant treatment reduces Angptl2-associated oxidative stress and decreases papilloma and SCC formation in a SCC model

We previously proposed that Angptl2 expression in skin tissues accelerates chemically induced carcinogenesis by increasing susceptibility to "pre-neoplastic change" and "malignant conversion" (7). To determine whether these changes are attributable to increased oxidative stress in those tissues, we treated mice with the potent antioxidant NAC by including it in their drinking water (25). Immunoblot analysis using anti-4-HNE antibody of skin tissues harvested 7 weeks after initiating chemical application indicated that levels of reactive oxygen species (ROS) in NAC-treated K14-Angptl2 Tg mice were significantly lower than those seen in Tg mice not treated with NAC (Fig. 3A and B). By 14 weeks
after initiation of chemical treatment, K14-Angptl2 Tg mice exhibited numerous papillomas in the absence of NAC treatment (Fig. 3C, left), whereas NAC-treated Tg mice showed a significantly reduced number of papillomas (Fig. 3C, right). The average latency is defined as time to reach 50% incidence of all papillomas (7, 26, 27). NAC-treated K14-Angptl2 Tg mice also showed attenuated formation of skin papillomas, with an average latency of 11 weeks after the beginning of chemical application, as compared with K14-Angptl2 Tg mice not treated with NAC, which showed an average latency of 7 weeks (Fig. 3D, top). In addition, K14-Angptl2 Tg mice treated with NAC showed significantly fewer papillomas (Fig. 3D, bottom). After 20 weeks of chemical treatment, NAC-treated K14-Angptl2 Tg mice exhibited an average of 9.2 papillomas per mouse, whereas K14-Angptl2 Tg mice without NAC exhibited an average of 26.5 (P < 1 × 10^{-3}). When only large papillomas (diameter > 3 mm) were evaluated, K14-Angptl2 Tg with treated with

Figure 3. NAC treatment reduces oxidative stress and susceptibility to chemical tumorigenesis in K14-Angptl2 Tg mice. A and B, Western blot analysis using the 4-hydroxy-2-nonenal (4-HNE) antibody to analyze skin tissues of K14-Angptl2 Tg mice provided drinking water without (left) or with (right) NAC. Mice were chemically treated as in Fig. 2. Hsc70 serves as an internal control. B, quantitative analysis of Western blot analysis (n = 3) of K14-Angptl2 Tg mice with and without NAC treatment (K14-Angptl2 Tg without NAC treatment data set at 1). Graphs show data in the 24 to 97 kDa range. Data are expressed as means ± SEM. **, P < 0.01 compared with K14-Angptl2 Tg mice given normal water. C, image showing skin of K14-Angptl2 Tg mice administered normal (left) and NAC-treated (right) water at 14 weeks after the first chemical application. D and E, top, “incidence” is defined as the percentage of mice with papillomas; bottom, the number of all detectable (1–3 mm) (D) or large (>3 mm) (E) papillomas from K14-Angptl2 Tg mice provided untreated (n = 20; red circles) or NAC-treated (n = 28; blue circles) water. P < 0.005 from weeks 6 to 20 (in D, bottom), P < 0.05 from weeks 10 to 20 (E, bottom). F, proportion of large papillomas seen in K14-Angptl2 Tg mice provided normal (Tg) or NAC-containing (Tg+NAC) water.
NAC developed papillomas 3 weeks later than did Tg mice without NAC treatment (Fig. 3E, top), and the average number of large papillomas decreased to 0.35-fold that seen in NAC-treated Tg mice (Fig. 3E, bottom). In addition, we performed the same experiments in wild-type mice and confirmed the tumor-inhibitory effect of NAC (Supplementary Fig. S2).

Interestingly, we observed no difference in the ratio of large to total papillomas between mice treated with or without NAC (Fig. 3F), whereas SCC formation was significantly attenuated in NAC-treated versus untreated Tg mice (compare right with left images in Fig. 4A). By 30 weeks after initiation of chemical treatment, 100% of K14-Angptl2 Tg without NAC treatment had developed malignant SCC, a condition seen in only 50% of NAC-treated mice (Fig. 4B, top). The average number of SCC tumors in NAC-treated K14-Angptl2 Tg decreased to 0.15-fold relative to untreated Tg mice (Fig. 4B, bottom). The malignant conversion rate, defined as the ratio of the number of SCCs to the number of large papillomas (7, 26, 27), in NAC-treated K14-Angptl2 Tg mice was lower than that in Tg mice without NAC treatment (Fig. 4C). In addition, survival of NAC-treated Tg mice was significantly prolonged relative to untreated Tg mice (Fig. 4D). Taken together, these results suggest that increased susceptibility to chemically induced skin carcinogenesis seen in this model is attributable to increased ROS production in skin tissues promoted by Angptl2 overexpression.
Decreased Msh2 mRNA levels in skin tissues of K14-Angptl2 Tg mice are ameliorated by NAC treatment

Because inflammation and oxidative stress downregulate DNA-repair MMR proteins (28, 29), we used quantitative real-time PCR to determine whether Angptl2 alters the expression of Msh2, which encodes a MMR protein. Msh2 expression level assessed prior to oncogenic chemical treatment differed in skin tissues of wild-type and K14-Angptl2 Tg mice (Fig. 5A). By 7 weeks after initiation of chemical application, Msh2 expression levels decreased in skin tissues of both K14-Angptl2 Tg and wild-type mice, although that decrease was significantly greater in tissues from Tg mice (Fig. 5A). In addition, we found that Msh2 expression in skin did not decrease in Angptl2 KO mice following chemical treatment (Supplementary Fig. S3).

Other DNA repair pathways such as nonhomologous end joining and homologous recombination could underlie rescue of DNA damage in chemical skin carcinogenesis. Therefore, we undertook Western blot analysis to assess levels of DNA-repair enzymes such as Atm and Atr (double- or single-strand breaks), Ku-70, and Ku-80 (nonhomologous end joining), and Rad51 and Brca2 (homologous recombination) in skin of wild-type, K14-Angptl2, and Angptl2 KO mice. We found that, except for Msh2, expression levels of almost all of these factors were similar between wild-type and Angptl2 KO mice or between Angptl2 Tg and wild-type mice (Supplementary Figs S4 and S5). These results suggest that the Angptl2-induced decrease in Msh2 protein levels likely plays an important role in carcinogenesis.

Next, we examined whether decreased Msh2 expression levels seen in K14-Angptl2 Tg were attributable to increased ROS production in skin tissues. As noted, at 7 weeks after initiation of chemical application, Msh2 expression levels in skin tissues of K14-Angptl2 Tg mice had significantly

Figure 5. Angptl2-dependent hypermethylation of the Msh2 promoter is ameliorated by antioxidant treatment. A and B, comparative expression levels of Msh2 in skin tissues (n = 7) following a single application of DMBA plus 0 or 7 applications of PMA in K14-Angptl2 Tg (Tg) versus wild-type (WT) mice (A), and between Tg mice provided normal (Tg) or NAC-treated (Tg + NAC) water (B). Values from wild-type or K14-Angptl2 Tg without NAC treatment at week 0 data were set at 1. Data are expressed as mean ± SEM. **, P < 0.01; n.s., not statistically significant. C and E, representative COBRA methylation analysis following a single application of DMBA plus 7 applications of PMA to skin tissues of K14-Angptl2 Tg or wild-type mice (C), and to K14-Angptl2 Tg mice provided normal (Angptl2 Tg) or NAC-treated (Angptl2 Tg + NAC) water (E). CpG-methylated NIH 3T3 mouse genomic DNA served as a positive control (P). NIH 3T3 mouse genomic DNA served as a negative control (N). D and F, methylation percentage seen in K14-Angptl2 Tg (n = 7) or wild-type (n = 7) mice (D), and in K14-Angptl2 Tg mice provided normal (Angptl2 Tg) (n = 10) or NAC-treated (Angptl2 Tg + NAC) water (n = 10) (F). Data are expressed as mean ± SEM. **, P < 0.01 compared with wild-type mice.
Methylation of the Msh2 promoter in skin tissues of K14-Angptl2 Tg mice decreases following NAC treatment

Oxidative stress has been associated with epigenetic modifications (30–32). Therefore, we asked whether decreased Msh2 expression seen in skin tissues of K14-Angptl2 Tg mice was due to oxidative stress and subsequent epigenetic modification of Msh2. To do so, we analyzed Msh2 promoter methylation using COBRA, which provides reliable quantitative results across several DNA methylation levels (17, 33–35). Analysis of restriction digestion patterns of genomic DNA isolated from skin tissues of K14-Angptl2 Tg mice indicated increased methylation frequency at the Msh2 promoter, relative to levels seen in wild-type mice (Fig. 5C). Quantitative analysis revealed that approximately 60% of tissue samples from K14-Angptl2 Tg mice showed high Msh2 promoter methylation, whereas those levels at the same region of the promoter were seen in only 6.9% of tissue samples from wild-type mice (Fig. 5D). Representative data shown in Fig. 5E indicates that Msh2 promoter methylation was significantly ameliorated by NAC treatment. Quantitative analysis revealed that NAC treatment decreased Msh2 promoter methylation in skin tissues of K14-Angptl2 Tg mice (Fig. 5F) to levels indistinguishable from those seen in wild-type mice (Fig. 5D). We conclude that decreased Msh2 mRNA expression in skin tissues of K14-Angptl2 Tg mice is likely attributable to Angptl2-induced oxidative stress and resultant increased Msh2 promoter methylation.

NAC treatment attenuates Angptl2-induced skin tissue inflammation in a chemically induced SCC model

We previously reported that, based on levels of the proinflammatory factors interleukin (IL)-1β and IL-6, Angptl2 expression levels in skin tissue are correlated with skin tissue inflammatory status in chemically induced SCC (7). Therefore, we asked whether NAC treatment altered IL-1β and IL-6 expression levels. IL-1β and IL-6 expression was significantly lower in the skin of NAC-treated compared with untreated Tg mice before chemical application (Fig. 5B). These findings suggest that NAC treatment decreases inflammation in skin tissues of Tg mice by suppressing ROS production, and that Angptl2-induced inflammation is enhanced by oxidative stress.

MSH2 levels are inversely correlated with ANGPTL2 expression in UV-exposed human skin tissues

Finally, we examined ANGPTL2 and MSH2 expression in UV-exposed human skin tissues. We found that ANGPTL2 expression was low, whereas MSH2 expression was robust in normal skin tissues with minimal sun exposure (Fig. 7A). Although ANGPTL2 expression fluctuated somewhat in cases of actinic keratosis, MSH2 expression was inversely correlated with ANGPTL2 expression (Fig. 7B; Supplementary Fig. S8). In addition, ANGPTL2 expression was robust and MSH2 expression was weak in tissue of sun-exposed squamous cell carcinoma (Fig. 7C). These results suggest that UV exposure induces ANGPTL2 expression, leading to decreases MSH2 levels in human skin tissues.

Discussion

Our previous findings that K14-Angptl2 Tg mice do not show papillomas or carcinomas when mice are treated with a tumor promoter (PMA) alone (7) indicate that Angptl2 is not an oncogene. However, we concluded that increased...
Angptl2 expression in skin tissue increases inflammation and accelerates carcinogenesis by enhancing susceptibility to both "pre-neoplastic change" and "malignant conversion." Our conclusion was based on studies using a skin carcinogenesis mice model employing DMBA/PMA treatment (7), a well-characterized model in which an initiating oncogenic mutation is followed by accumulation of additional oncogenic mutations (8–10). Findings reported here suggest that Angptl2 expression induces skin inflammation and likely accelerates acquisition of oncogenic mutations resulting in carcinogenesis. In addition, we find that in skin tissues Angptl2 overexpression correlates with decreased Msh2 expression, likely due to methylation of its promoter region triggered by ROS accumulation in those tissues. Conversely, we show that continuous treatment with the antioxidant NAC reduces ROS accumulation in skin tissues and ameliorates decreased Msh2 expression due to epigenetic modification, decreasing susceptibility to carcinogenesis. Finally, we report that antioxidant treatment also decreases skin tissue inflammation in K14-Angptl2 Tg mice by suppressing ROS production, suggesting that Angptl2-induced inflammation is enhanced under oxidative stress conditions. Overall, we conclude that enhanced oxidative stress and chronic inflammation induced by Angptl2 synergize to increase susceptibility for carcinogenesis.

Currently, we do not know the mechanism underlying enhanced ROS production in skin tissue expressing excess Angptl2. Our previous report showing chronic inflammation in skin tissue of K14-Angptl2 Tg mice demonstrated infiltration of skin tissue by inflammatory cells, including activated macrophages and neutrophils (7), both of which are sources of ROS (36). Angptl2 reportedly activates NF-kB proinflammatory signaling in various cell types (3–7, 37), which also enhances ROS production (38). Taken together, Angptl2 secreted from skin tissue of K14-Angptl2 Tg mice may increase ROS production due to activity of infiltrated and activated inflammatory cells.

In addition, we demonstrate here decreased Msh2 expression in skin tissues of K14-Angptl2 Tg mice, most likely due to methylation of the Msh2 promoter, an activity significantly improved by NAC treatment. Thus increased oxidative stress caused by Angptl2 overexpression may promote Msh2 promoter methylation. Previously, others have reported that ROS promotes development of human carcinogenesis through epigenetic regulation of gene expression (32). For example, ROS-induced oxidative stress reportedly silences promoters of tumor suppressors via hypermethylation (39). In addition, ROS induces Snail, which activates histone deacetylase 1 and DNA methyltransferase 1 (40). Moreover, we have

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**Figure 7.** ANGPTL2 and MSH2 expression levels in normal skin, actinic keratosis, and squamous cell carcinoma. A–C, representative photographs of hematoxylin and eosin staining and immunohistochemical analysis with ANGPTL2 and MSH2 antibodies in normal human abdominal skin (non–sun-exposed area) (A), actinic keratosis on the face (sun-exposed area) (B), and SCC on the face (sun-exposed area) (C). Scale bars, 500 μm.
reported induction of an epithelial-to-mesenchymal transition (EMT) by increasing Snail mRNA expression and activating the TGF-β pathway in SCC of K14–Angptl2 Tg mice (7), suggesting that Snail functions in Msh2 promoter methylation. Further, ROS promotes phosphorylation of the transcription factors c-Jun and ATF2, increasing expression of their target genes (41). Interestingly, we reported that c-Jun and ATF2 activity increases Angpt2 expression (6), suggesting a mechanism through which ROS might increase Angpt2 expression.

We conclude that Angpt2 accelerates susceptibility to both "preneoplastic change" and "malignant conversion" by activating a cycle of chronic inflammation and oxidative stress in the premalignant tissue microenvironment (Supplementary Fig. S9). More recently, we observed that spontaneous carcinogenesis is significantly decreased in Angptl2 KO compared with wild-type mice (our unpublished data), suggesting that Angpt2 contributes not only to chemically induced carcinogenesis but also to various types of spontaneous cancers. Overall, our findings suggest that Angpt2 represents a target to develop new strategies to antagonize these activities in premalignant tissue.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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