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 protein2 (Angptl2) facilitates inflammatory carcinogenesis and metastasis in a chemically-induced skin squamous cell carcinoma (SCC) 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 those tissues. Conversely, in the context of this model, Angptl2 knockout (KO) mice show significantly decreased oxidative stress in skin tissue and lower incidence of SCC compared to wild-type mice. In the chemically-induced SCC model, treatment of K14-Angptl2 Tg mice with the anti-oxidant N-acetyl cysteine (NAC) significantly reduced oxidative stress in skin tissue and 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 to 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, and 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 nineteenth century (1). Since then epidemiological studies have confirmed the idea that chronic inflammation underlies many cancers (2). Recently, we reported that Angiopoietin-like protein2 (Angptl2) acts as a chronic inflammatory mediator in several pathological settings (3-6) and demonstrated that increased Angptl2 expression in skin tissues promotes inflammation and accelerates carcinogenesis in a chemically-induced skin squamous cell carcinoma (SCC) 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 mismatch repair (MMR) family members, which correct base mispairings or repair larger insertion/deletion loops (IDLs) (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 increases 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
the 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 anti-oxidant 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-h 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 mM) was administered in drinking water from 7 weeks after birth until the end of 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 the above groups with or without NAC treatment. All experiments were performed according to 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, St Louis, MO) was applied topically to the shaved back skin of 8-wk-old male mice, followed by weekly topical application of 5 μg of the tumor promoter phorbol ester 12-O-tetradecanoylphorbol 13-acetate (PMA; Sigma, St Louis, MO) 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 the following 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. On the other hand, SCCs are invasive carcinomas of the surface epidermis. SCCs proliferate download 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's 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 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM EDTA, 300 mM NaCl, 50 mM Tris-HCl, pH 7.5, and 1% Triton X-100). Extracts derived from supernatants were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using an ECL kit (Amersham, Buckinghamshire, UK) according to the manufacturer’s protocol. Antibodies used were against 4-hydroxy-2-nonenal (4-HNE) (Santa Cruz Biotechnology, Santa Cruz, CA), Hsc70 (B-6) (R&D Systems, Minneapolis, MN), Rad51, Brca2, Atm, Atr, Ku70, Ku80, and Msh2 (Cell Signaling Technology, Danvers, MA) and Angptl2 (3).
Immunohistochemical staining

Skin tissues were fixed by perfusion with 4% paraformaldehyde in PBS (pH 7.4), washed in PBS for 15 min, 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 mM periodic acid for 10 min at room temperature to inhibit endogenous peroxidase. Specimens were incubated for 1 h with 50–100-fold diluted polyclonal antibody against Angptl2 (3) or Msh2 (Cell Signaling), and then washed three times with PBS for 5 min. Sections were incubated with biotinylated anti-mouse IgG or anti-rabbit IgG (1:200 dilution) (Vector Laboratories, Burlingame, CA). Immunostaining was performed using the peroxidase-labeled avidin-biotin-complex method (1:100 dilution) (DAKO, Glostrup, Denmark). 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 bisulfite 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 COBRA assay (Combined Bisulfite Restriction Analysis) (17). Bisulfite treatment of genomic DNA was performed using the MethylEasy™ Xceed Rapid DNA Bisulphite Modification Kit (Human Genetic Signatures, Sydney, Australia) according to manufacturer’s instructions. CpG methylated NIH 3T3 mouse genomic DNA (New England Biolabs, Ipswich, MA) served as a positive control and underwent bisulfite modification as described above. NIH 3T3 mouse genomic DNA (New England Biolabs, Ipswich, MA)
served as a negative control. The proportion of methylated versus unmethylated product (digested versus undigested) was quantified by densitometry (23). Densitometric analysis was performed using Multi Gauge software (Fujifilm, Tokyo, Japan).

**PCR and restriction digestion.**

Msh2 primers were specially designed for the COBRA assay as described (17). Sequences used were 5’- TGGCGGTGTAGTTTAAGGAGA -3’ (forward) and 5’- TCTTAAACACCTCGCGAACC -3’ (reverse), generating a 195 bp amplification product (17). PCR amplifications were performed using TaKaRa EpiTaq™ HS (for bisulfite-treated DNA) (Takara Bio, Otsu, Japan) according to the manufacturer's instructions. After amplification, PCR products were digested with MboI (Promega, Madison WI, USA), 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 versus undigested) was quantified by densitometry (17, 23) using MultiGauge version 3.1 software (Fuji Film, Tokyo, Japan).

**Calculation of survival data.**

A Kaplan-Meier log-rank test was applied to analyze mouse survival data using JMP7 software (SAS Institute, Cary, NC). 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 TRIzol reagent (Invitrogen, Carlsbad, CA). DNase-treated RNA was reverse transcribed with a PrimeScript RT reagent Kit (Takara Bio, Otsu, Japan). PCR products were analyzed with a Thermal Cycler Dice Real Time system (Takara Bio, Otsu, Japan), and relative transcript abundance was normalized to that of 18S mRNA. PCR oligonucleotides were as follows: mouse $\text{Msh2}$: forward, 5'-GCCCAGGATGCCATTGTTAAAG-3'; reverse, 5'-AACGGGTGCTGCGTTTGAC-3'; mouse $\text{18S}$: forward, 5'-TTCTGGCCAACGGTCTAGACAAC-3'; reverse, 5'-CCAGTGGTCTTGGTGTGCTGA-3'; mouse $\text{IL-1}\beta$: forward, 5'-TCCAGGATGAGGACATGAGCAC-3'; reverse, 5'-GAACGTCACACACCAGGCTTA-3'; mouse $\text{IL-6}$: forward, 5'-CCACTTCACAAGTCGGAGGCTTA-3'; reverse, 5'-GCAAGTGCATCATCGTTGTTCATAC-3'. Results were analyzed using the 2-tailed Student’s $t$-test with Excel software (Microsoft, Redmond, WA). 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 carcinogenesis among K14-Angptl2 Tg, wild-type and Angptl2 KO mice by estimating the frequency and time course of SCC development in mice of all three genotypes. As we previously reported, increased Angptl2 expression in skin tissues accelerated carcinogenesis in the SCC model (Fig. 1A), while Angptl2 loss antagonized carcinogenesis (Fig. 1B). Seven weeks after initiation of chemical treatment, K14-Angptl2 Tg, Angptl2 KO, and respective wild-type mice showed little evidence of papillomas or SCC in chemically-treated skin 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, B). Conversely, levels of HNE-modified proteins in skin tissues of Angptl2 KO mice were
significantly decreased compared to wild-type controls (Fig.2C, D). We also found that HNE-modified protein levels were positively correlated with the Angptl2 levels (Fig 2A and 2C). These findings suggest that Angptl2 increases oxidative stress.

**Anti-oxidant 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, N-acetyl cysteine (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 ROS levels in NAC-treated K14-Angptl2 Tg mice were significantly lower than those seen in Tg mice not treated with NAC (Fig.3A, B). By 14 weeks after initiation of chemical treatment, K14-Angptl2 Tg mice exhibited numerous papillomas in the absence of NAC treatment (left image in Fig. 3C), while NAC-treated Tg mice showed a significantly reduced number of papillomas (right image in Fig. 3C). The average latency is defined as time to reach 50% incidence of all papillomas (7, 26, 27). NAC-treated K14-Angptl2 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 (upper graph in Fig. 3D). K14-Angptl2 Tg mice treated with NAC
also showed significantly fewer papillomas (lower graph in Fig. 3D). After 20 weeks of chemical treatment, NAC-treated K14-Angptl2 Tg mice exhibited an average of 9.2 papillomas per mouse, while K14-Angptl2 Tg mice without NAC exhibited an average of 26.5 ($p < 1 \times 10^{-9}$). When only large papillomas (diameter > 3mm) were evaluated, K14-Angptl2 Tg with treated with NAC developed papillomas 3 weeks later than did Tg mice without NAC treatment (upper graph in Fig. 3E), and the average number of large papillomas decreased to 0.35-fold that seen in NAC-treated Tg mice (lower graph in Fig. 3E). 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 (upper graph in Fig. 4B). The average number of SCC tumors in NAC-treated K14-Angptl2 Tg decreased to 0.15-fold relative to untreated Tg mice (lower graph in Fig. 4B). 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 in Tg mice without NAC treatment (Fig. 4C). Survival of NAC-treated Tg mice was also 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.**

Since inflammation and oxidative stress down-regulate DNA repair MMR proteins (28, 29), we used quantitative real-time PCR to determine whether Angptl2 alters 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). We also 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 non-homologous end joining and homologous recombination could underlie rescue of DNA damage in chemical skin carcinogenesis. Therefore, we undertook western analysis to assess levels of DNA-repair enzymes such as Atm and Atr (double- or single-strand breaks), Ku-70 and Ku-80 (non-homologous end joining), and Rad51 and Brca2 (homologous recombination) in skin of wild-type, K14-Angptl2, and Angptl2 KO mice. We found that, except for except Msh2, expression levels of almost all of these factors were almost same between wild-type and Angptl2 KO mice or between Angptl2 Tg and wild-type mice (Supplementary Fig. 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 decreased, whereas those decreases were ameliorated in Tg mice treated with NAC (Fig. 5B and Supplementary Fig S6). Protein levels of DNA repair enzymes, except Msh2, were unchanged by NAC treatment (Supplementary Fig S6). In addition, NAC treatment itself did not increase Msh2 transcript levels (Supplementary Fig S7), and decreased Msh2 expression was ameliorated by NAC treatment of both wild-type and K14-Angptl2 Tg mice at 7 weeks after initiation of chemical application (Fig. 5B and Supplementary Fig. S7). We conclude that high Angptl2 levels may suppress Msh2 expression in skin tissues by increasing ROS production.

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 combined bisulfite restriction analysis (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 Figure 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 pro-inflammatory factors 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 to untreated Tg mice before chemical application (Fig. 6). 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, while 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 and 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-κB pro-inflammatory 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.

We also 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). ROS also induces Snail, which activates histone deacetylase 1 and DNA methyltransferase 1 (40). We have also 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. ROS also 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 Angptl2 expression (6), suggesting a mechanism through which ROS might increase Angptl2 expression.

We conclude that Angptl2 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 to wild-type mice (our unpublished data), suggesting that Angptl2 contributes not only to chemically-induced carcinogenesis but also to various types of spontaneous cancers. Overall, our findings suggest that Angptl2 represents a target to develop new strategies to antagonize these activities in premalignant tissue.
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Disclosure

Authors declare no conflict of interest.
References


29. Hsu T, Huang KM, Tsai HT, Sung ST, Ho TN. Cadmium(Cd)-induced oxidative stress down-regulates the gene expression of DNA mismatch recognition proteins MutS homolog 2 (MSH2) and MSH6 in zebrafish (Danio rerio) embryos. Aquat Toxicol 2013;126:9-16.


36. Klaunig JE, Kamendulis LM. The role of oxidative stress in...


Figure Legends

Figure 1. Chemical skin carcinogenesis is accelerated in skin tissues constitutively expressing Angptl2 and suppressed in Angptl2-deficient tissues. (A, B) Representative photographs showing the same mouse (in each row) throughout the time course of chemical skin carcinogenesis from both K14-Angptl2 Tg and wild-type littermates (A) and Angptl2 KO and wild-type littermates (B) at 10 (left), 15 (middle), and 20 (right) weeks after initiation of chemical application.

Figure 2. ROS levels increase in skin tissues of K14-Angptl2 transgenic mice and decrease in tissues from Angptl2 KO mice. (A, C) Western blotting analysis using 4-hydroxy-2-nonenal (4-HNE) antibody, which recognizes ROS-modified proteins, of skin tissues of K14-Angptl2 Tg and wild-type mice (A) and Angptl2 KO and wild-type mice (C). Skin tissues received a single application of DMBA plus 7 applications of PMA. Hsc70 serves as an internal control. (B, D) Quantitation of western blotting analysis (n = 3) in (B) and (D) (wild-type data set at 1). Graphs show data in the 24 to 97 kDa range. Data are expressed as means ± s.e.m. **P < 0.01 compared with wild-type mice.

Figure 3. N-acetyl cysteine (NAC) treatment reduces oxidative stress and susceptibility to chemical tumorigenesis in K14-Angptl2 Tg mice. (A, B) Western blotting 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 Figure 2. Hsc70
serves as an internal control. (B) Quantitative analysis of western blotting 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 ± s.e.m. **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, E) (Upper panels) “Incidence” is defined as the percentage of mice with papillomas. (Lower panels) indicate 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 (lower graph in D), P < 0.05 from weeks 10 to 20 (lower graph in E). (F) Proportion of large papillomas seen in K14-Angptl2 Tg mice provided normal (Tg) or NAC-containing (Tg + NAC) water.

Figure 4. Antioxidant treatment prevents carcinogenesis and prolongs survival of K14-Angptl2 Tg mice. (A) Photograph of SCC exhibited by K14-Angptl2 Tg mice provided normal (left) or NAC-treated (right) water at 8 weeks after first diagnosis of SCC. Arrowheads indicate SCC. (B) Increased incidence of SCC (upper graph) and number of SCC tumors per mouse (lower graph, P < 0.05 after week 19) seen in K14-Angptl2 Tg mice administered normal (n = 20; red circles) or NAC-containing (n = 28; blue circles) water. (C) Comparison of the malignant conversion ratio of large papillomas to SCC in K14-Angptl2 Tg mice provided normal versus NAC-treated water. (D) Kaplan-Meier survival curves after initiation of chemical application of...
K14-Angptl2 Tg mice provided normal (n = 28) or NAC-treated (n = 27) water (P < 0.001 by log-rank test).

**Figure 5.** Angptl2-dependent hypermethylation of the Msh2 promoter is ameliorated by antioxidant treatment. (A, 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 means ± s.e.m. **P < 0.01, n.s., not statistically significant. (C, 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, 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 means ± s.e.m. **P < 0.01 compared with wild-type mice.

**Figure 6.** NAC treatment suppresses IL-1β and IL-6 expression in skin tissues following chemical treatment. (A, B) Comparative expression levels of IL-1β (A) and IL-6 (B) (n = 7) after a single application of DMBA plus 0 or 7 applications of PMA to skin tissues of K14-Angptl2 Tg mice provided normal
(Tg) or NAC-treated (Tg + NAC) water. Values from K14-Angptl2 Tg without NAC treatment at week 0 data were set at 1. Data are expressed as means ± s.e.m. **P < 0.01, n.s., not statistically significant.

Figure 7. ANGPTL2 and MSH2 expression levels in normal skin, actinic keratosis, and squamous cell carcinoma. (A, B, C) Representative photographs of H&E 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.
Figure 6 (Aoi et al.)
Figure 7 (Acl et al)
Angiopoietin-like Protein 2 Accelerates Carcinogenesis by Activating Chronic Inflammation and Oxidative Stress

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