MK2 regulates the early stages of skin tumor promotion

Claus Johansen, Christian Vestergaard, Knud Kragballe, George Kollia, Matthias Gaestel and Lars Iversen

Department of Dermatology, Aarhus University Hospital, P.O. Oerumsgade 11, 8000 Aarhus, Denmark. Institute of Immunology, Biomedical Sciences Research Center “Alexander Fleming”, 16672 Vari, Greece and Institute of Biochemistry, Hannover Medical School, 30625 Hannover, Germany

To whom correspondence should be addressed. Tel: +45 89491848; Fax: +45 89491850; Email: lars.iversen@ki.au.dk

The association between inflammation and tumorigenesis is well recognized. Mitogen-activated protein kinase-activated protein kinase-2 (MK2) is known to play a pivotal role in inflammatory processes. Here, we studied the effect of MK2-deficiency and tumor necrosis factor (TNF-α)-deficiency on skin tumor development in mice using the two-stage chemical carcinogenesis model. We found that MK2+/− mice developed significantly fewer skin tumors compared with both TNF-α−/− and wild-type mice when initiated by induction with 7,12-dimethylbenz[a]anthracene (DMBA) and by promotion with 12-O-tetradecanoylphorbol-13-acetate (TPA). The TPA-induced inflammatory response was reduced in both, TNF-α−/− mice and MK2+/− mice, but most pronounced in TNF-α−/− mice, indicating that a reduced inflammatory response was not the only explanation for the inhibited tumorigenesis seen in MK2+/− mice. Interestingly, increased numbers of apoptotic cells were detected in the epidermis of MK2+/− mice compared with TNF-α−/− and wild-type mice, suggesting an additional role of MK2 in the regulation of apoptosis. This was further supported by: (i) increased levels of the tumor suppressor protein p53 in MK2+/− mice after DMBA/TPA treatment compared with controls, (ii) reduced phosphorylation (activation) of the negative p53 regulator, murine double minute 2 in MK2+/− mouse keratinocytes in vitro and (iii) a significant decrease in the DMBA/TPA induced apoptosis in cultured MK2+/− keratinocytes transfected with p53 small interfering RNA. Taken together, these findings demonstrate a dual role of MK2 in the early stages of tumor promotion through regulation of both the inflammatory response and apoptosis of DNA-damaged cells. These results also identify MK2 as a putative target for future skin cancer therapy.

Introduction

Human skin cells are continuously exposed to genotoxic stress, which may result in DNA damage. Therefore, a network of DNA surveillance systems has evolved in the cells such as DNA repair and signaling pathways that activate cell cycle checkpoints. In cases where the DNA damage is too extensive, the cells undergo apoptosis (1). In general, the carcinogenesis process involves the distinct phases of initiation, promotion and progression. The tumor initiation stage is a process in which carcinogens damage the DNA and induce mutations in critical genes in stem cells. During tumor promotion, the malignant cells are stimulated to grow whereas tumor progression is the process in which the growing tumor becomes more aggressive (2–5).

The development of cancer in various organs is strongly associated with chronic infection and inflammation, suggesting a relationship between inflammation and carcinogenesis (3,6–9). Tumor necrosis factor (TNF-α) is a proinflammatory cytokine known to be a key downstream mediator in inflammation. Despite its name, TNF-α is very important in the early stages of tumor promotion, regulating a number of cytokines, chemokines, adhesion proteins, matrix metalloproteinases and pro-angiogenic activities (10). In support of this, mice deficient in TNF-α were demonstrated to be partially resistant to chemical-induced skin carcinogenesis. Furthermore, it was demonstrated that TNF-α had little influence on later stages of carcinogenesis, as tumors in TNF-α knockout mice and wild-type mice had similar rates of malignant progression (11).

The mitogen-activated protein kinase-activated protein kinase-2 (MK2) is a serine/threonine kinase regulated through direct phosphorylation by p38 mitogen-activated protein kinase (MAPK) (12). MK2 has been demonstrated to regulate TNF-α expression at a posttranscriptional level during an inflammatory response (13). MK2 knockout mice were shown to survive lipopolysaccharide-induced endotoxic shock due to a reduction of ~90% in the production of TNF-α (14). Furthermore, we have demonstrated that transfection of cultured human keratinocytes with MK2 small interfering RNA (siRNA) led to a reduction in the anisomycin-induced protein expression of TNF-α and interleukin (IL)-6 (13). MK2 signaling is known to increase TNF-α through translational control via the adenylyl uridylate-rich elements of the 3′-untranslated region of TNF-α messenger RNA (mRNA) (15) and to increase the mRNA stability of IL-6 (15,16).

The p53 protein is well known for its tumor suppressor function. After activation by stresses such as DNA damage and oncogenic insults, posttranslational modifications of p53 cause elevated protein levels and increased transcriptional activity, resulting in cellular changes such as cell-cycle arrest, DNA repair or apoptosis (17,18). A key player in the p53 network is the p53 inhibitor murine double minute 2 [(Mdm2); human double minute 2 in humans], which is a p53 target gene and a crucial negative regulator of p53, as the embryonic lethality in Mdm2-deficient mice is fully rescued by the absence of p53 (19–23). When activated, Mdm2 ubiquitinates p53, thereby targeting it for rapid degradation via the 26S proteasome (24–26). Recently, it was demonstrated that MK2 can phosphorylate Mdm2 and that MK2-deficient mouse embryonic fibroblasts have reduced Mdm2 phosphorylation and elevated protein levels of p53, suggesting that MK2 play a role in moderating the extent and duration of the p53 response (27). MK2 may therefore play a role in the regulation of both inflammation and apoptosis.

The potential role of MK2 in carcinogenesis has not been explored so far. The purpose of this study was to characterize the role of MK2 in tumor initiation and in the early stages of tumor promotion using the two-stage chemical skin carcinogenesis model in MK2-deficient mice. Our results demonstrate for the first time that MK2 plays a critical role in the development of skin tumors, not only by regulation of the inflammatory response but also by maintaining the survival of DNA-damaged cells induced by 7,12-dimethylbenz[a]anthracene (DMBA) at the time of tumor initiation.

Materials and methods

Mice

MK2 knockout mice carrying a mutation in the catalytic domain of the MK2 gene and TNF-α knockout mice were derived as described previously (14,28). The knockout mice and the wild-type mice were on a C57BL/6 genetic background.

Skin carcinogenesis

In each experiment, 6-week-old female mice were used. A dorsal area of 1–2 cm² skin was shaved and the mice were treated with a single application of DMBA (25 μg in 100 μl acetone; Sigma–Aldrich, St Louis, MO) followed...
by applications of 12-O-tetradecanoylphorbol-13-acetate (TPA) (4 μg in 100 μl aceton; Sigma–Aldrich) three times weekly for 15 weeks. The area was regularly shaved and the mice were observed daily. Tumor formation was assessed weekly, and tumors were defined as raised lesions of a minimum diameter of 1 mm that had been present for at least 2 weeks. Mice were monitored for 26 weeks. At the end of the study, mice were killed and tumors were removed and embedded in paraffin for histological analysis. Work was approved by the Danish Laboratory Animal Research committee. Mice were housed in specific pathogen-free conditions in accordance with European Union regulations.

Generation of keratinocyte cultures and cell culturing

Both wild-type keratinocytes and MK2−/− and TNF-α−/− keratinocytes were obtained from 5- to 6-week-old female mice epidermis. Briefly, the skin was removed from the dorsal area of the mice and washed in phosphate-buffered saline. The skin was incubated in Trypsin 0.25% (Gibco, Invitrogen, Carlsbad, CA; cat no. 25050-014) added 0.07 mM ethylenediaminetetraacetic acid for 2 h at 35°C. Then, the epidermis was separated from the dermis and incubated for 20 min on a vibrating table before filtered through a piece of 70 μm Teflon mesh. The keratinocytes were then isolated by centrifugation at 160g for 10 min at 4°C, resuspended and cultured in keratinocyte growth medium (CnT-07; CELLiNTEC Advanced Cell Systems, Bern, Switzerland) at 35°C and 5% CO2 in an incubator.

Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay

Apoptosis was detected with fluorescein-based labeling of DNA strand breaks using the DeadEnd Fluorometric Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) system (Promega, Madison, WI) according to the supplier’s instructions. Briefly, 4 sections of paraffin-embedded skin samples from MK2−/−, TNF-α−/− and wild-type mice were used. The samples were deparaffinized by immersing the samples in xylene after which the samples were rehydrated by sequentially immersing the samples through graded alcohols. The sections were washed before embedding with paraffin and the samples were deparaffinized by incubating the sections in xylene at 60°C for 1 h. After which, the samples were centrifuged at 10000g for 10 min at 4°C, resuspended and cultured in keratinocyte growth medium (CnT-07; CELLiNTEC Advanced Cell Systems, Bern, Switzerland) at 35°C and 5% CO2 in an incubator.

Carcinogenesis

MK2 is essential in skin tumor promotion

Results

MK2−/− mice are resistant to skin tumor development

To assess the role of MK2 in multistage skin carcinogenesis, MK2-deficient mice, TNF-α-deficient mice and wild-type mice were subjected to a single topical application of DMBA (25 μg), followed one week later by topical application of 4 μg TPA three times a week for 15 weeks. Papillomas were first observed in the wild-type mice (n = 14) 8 weeks after the start of TPA treatment, and by week 18, 93% of these mice had developed an average of 5.4 papillomas per mouse (Figure 1A–C). In contrast, no papillomas were found in the MK2−/− mice (n = 15) until 12 weeks after the start of TPA promotion, and by week 18, only 7% (one mouse out of 15) had developed one papilloma giving an average of 0.07 papillomas per mouse. The difference in papilloma incidence between wild-type and MK2−/− mice was statistically significant (P < 0.001) from week 8 and onwards. TNF-α−/− mice have previously been demonstrated to be partially resistant to skin carcinogenesis (11,30). In agreement with these studies, we found that TNF-α−/− mice (n = 11) developed significantly fewer papillomas compared with wild-type mice (P = 0.001, week 18). Papillomas were first found in TNF-α−/− mice 11 weeks after the start of promotion, and by week 18, 46% of these mice had developed skin papillomas, giving an average of 1.1 papillomas per mouse (Figure 1A–C). Interestingly, statistically significant (P = 0.012) more papillomas were seen in TNF-α−/− mice compared with MK2−/− mice at week 18. Histological assessment of the skin tumors from wild-type and TNF-α−/− mice revealed that 29% of the tumors from wild-type and 20% of the tumors from TNF-α−/− mice were scored as carcinomas, whereas the remaining proportion was scored as benign with no invasion. The single tumor from MK2−/− mice, which was available for histological examination, was scored as a carcinoma (supplementary Table 1 is available at Carcinogenesis Online). We also examined the role of MK2 and TNF-α in TPA-induced hyperproliferation, but no significant changes in the thickness of the epidermis after treatment with DMBA/TPA were observed between MK2−/−, TNF-α−/− and wild-type mice (supplementary Figure 1 is available at Carcinogenesis Online).
Taken together, these results indicate a critical role for MK2 in the development of papillomas in mouse skin, using the DMBA/TPA carcinogenesis model. Because significantly fewer tumors were seen in MK2/C0/C0 mice compared with TNF-α/C0/C0 mice, the protective effect of MK2-deficiency cannot solely be explained by reduced TNF-α expression.

DMBA/TPA-induced IL-1β, IL-6 and TNF-α mRNA and protein expression are impaired in MK2−/− and TNF-α−/− mice

To characterize the inflammatory response in DMBA/TPA-induced tumor formation, IL-1β, IL-6 and TNF-α mRNA expression as well as protein levels were analyzed in wild-type, MK2−/− and TNF-α−/− mice by quantitative PCR and ELISA, respectively. DMBA/TPA treatment of wild-type mice led to an ~150-fold increase in IL-1β mRNA expression after 2 h compared with vehicle-treated wild-type mice (Figure 2A). At the same time point, the increase in IL-1β mRNA expression was significantly inhibited in both MK2−/− and TNF-α−/− mice (P = 0.012 and P = 0.009, respectively). No significant differences in IL-1β mRNA expression were seen between MK2−/− and TNF-α−/− mice. ELISA experiments demonstrated that 6 and 24 h after the last TPA treatment, the protein level of IL-1β was significantly decreased in MK2−/− and TNF-α−/− mice compared with wild-type mice (6 h: P = 0.026 and P = 0.03, respectively, and 24 h: P = 0.027 and P = 0.026, respectively) (Figure 2A).

DMBA/TPA treatment increased the level of TNF-α mRNA by ~14-fold after 2 h in wild-type mice compared with vehicle-treated mice. The induction in TNF-α mRNA expression was significantly reduced in MK2−/− mice after both 2 and 6 h (P = 0.016 and P = 0.034, respectively) (Figure 2B). As expected no TNF-α mRNA was detected in TNF-α−/− mice. Also, the TNF-α protein level was indistinguishable from the basal level of vehicle-treated mice in MK2−/− mice 24 h after the last TPA treatment, whereas in wild-type mice, a significant increase in TNF-α protein was detected (Figure 2B).

IL-6 mRNA expression was also dramatically increased in wild-type mice after DMBA/TPA treatment, resulting in an increase of ~150-fold after 2 h. In both MK2−/− and TNF-α−/− mice, the DMBA/TPA-induced IL-6 mRNA expression after 2 h was significantly impaired compared with treated wild-type mice (P = 0.018 and P = 0.025, respectively) (Figure 2C). The IL-6 protein level was significantly decreased in both MK2−/− and TNF-α−/−...
mice 6 h after the last TPA treatment compared with wild-type mice ($P = 0.032$ and $P = 0.015$, respectively) (Figure 2C). Finally, we examined the expression of the anti-inflammatory cytokine IL-10. Although the expression of IL-10 was increased after DMBA/TPA treatment, no changes in the mRNA expression and protein levels were found between treated wild-type mice and treated MK2$^{−/−}$ and TNF-$\alpha$<sup>−/−</sup> mice (Figure 2D).

**Decreased MPO levels in MK2$^{−/−}$ and TNF-$\alpha$<sup>−/−</sup> mice**

Mice deficient in MK2 or TNF-$\alpha$ had an impaired inflammatory response as analyzed by decreased mRNA and protein expression of proinflammatory cytokines in the skin. To further characterize the inflammatory response in the skin, the MPO activity was determined to assess the numbers of neutrophils. One application of TPA resulted in a significant increase in MPO levels in biopsies from

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**Fig. 2.** The mRNA expression and protein level of IL-1$\beta$, TNF-$\alpha$, and IL-6 are decreased in both MK2$^{−/−}$ and TNF-$\alpha$<sup>−/−</sup> mice following treatment with DMBA/TPA. (A-D) Groups of mice ($n = 7$) were treated with 25 µg DMBA followed 1 week later by four applications of TPA (4 µg) or vehicle (Veh) over 7 days. After the last application of TPA, the mice were killed and punch biopsies were taken at the indicated time points and analyzed by quantitative reverse transcription-polymerase chain reaction or ELISA for mRNA and protein levels of (A) IL-1$\beta$, (B) TNF-$\alpha$, (C) IL-6, and (D) IL-10. The mRNA expression of IL-1$\beta$, TNF-$\beta$, IL-6 and IL-10 was normalized to GAPDH mRNA expression. Results represent mean (±SE) and are representative of at least three independent experiments. All measurements were performed in triplicates. *$P < 0.05$ compared with wild-type mice treated for 24 h. **$P < 0.05$ compared with wild-type mice treated for 6 h. $\#P < 0.05$ compared with veh-treated wild-type mice.
wild-type skin compared with vehicle-treated skin, reflecting elevated neutrophilic cell infiltration into the skin (Figure 3A). In contrast, the MPO levels in TPA-treated skin from both MK2−/− and TNF-a−/− mice were significantly decreased compared with TPA-treated skin from wild-type mice (P<0.002 and P<0.0001, respectively). Interestingly, a significantly lower MPO level was seen in TNF-a−/− mice compared with MK2−/− mice (P<0.01), indicating that a reduced inflammatory response was not the only explanation for the inhibited tumorigenesis seen in MK2−/− mice. In addition, histological analysis was conducted to visualize inflammatory granulocyte infiltrates in the skin of wild-type mice treated with or without DMBA/TPA. Increased numbers of inflammatory granulocytes were seen in the dermis of DMBA/TPA-treated mice compared with vehicle-treated mice (Figure 3B).

MK2-deficient mice are more sensitive to DMBA/TPA-induced apoptosis
To study a possible effect of MK2-deficiency on apoptosis in mice skin, a TUNEL assay measuring the number of apoptotic cells in the epidermis was conducted. One application of DMBA followed 1 week later by four applications of TPA over 7 days significantly increased the number of apoptotic cells in the epidermis of MK2−/− mice as compared with wild-type mice (P = 0.001) (Figure 4A). In contrast, no difference in the number of apoptotic cells between TNF-a−/− and wild-type mice was seen. Also, the number of apoptotic cells in vehicle-treated epidermis was found to be significantly higher in MK2−/− mice compared with wild-type mice (P = 0.004). Examination of the TUNEL-positive cells revealed that the majority of DMBA/TPA-induced apoptotic cells were located in the region of the hair.
of apoptotic cells per square millimeter epidermis (±SE). Cells, 4 mm punch biopsies were taken and embedded in paraffin. Skin sections were then stained by the TUNEL method. Results are shown as the number of apoptotic cells per square millimeter epidermis (±SE). *P < 0.01 compared with wild-type mice. (C) Staining of apoptotic cells (TUNEL; green) and cell nuclei (4′,6-diamidino-2-phenylindole; blue) in skin of MK2−/− mice treated with DMBA/TPA. (D) Five mice in each group were treated with DMBA (25 μg) or vehicle followed 1 week later by four applications of TPA (4 μg) or vehicle over 7 days. Twenty-four hours after the last TPA application, whole cell extracts were isolated from skin biopsies taken from the back of the mice and analyzed by western blotting using antibodies directed against cleaved caspase-3 and β-actin. Scale bar, 25 μm; arrows indicate TUNEL-positive cells.

To confirm that TUNEL-positive cells do reflect increased apoptosis in the skin, caspase-3 activity was measured. Mice were given one application of DMBA for 24 h to the back of the mice, did not result in any difference in the number of apoptotic cells between vehicle- and DMBA-treated MK2−/− mice, whereas elevated numbers of apoptotic cells were seen in DMBA/TPA-treated MK2−/− skin compared with vehicle-treated MK2−/− skin and DMBA-treated MK2−/− skin (Figure 4A and B). No difference in the number of TUNEL-positive cells between TNF-α−/− and wild-type mice was found.

To confirm that TUNEL-positive cells do reflect increased apoptosis in the skin, caspase-3 activity was measured. Mice were given one application of DMBA for 24 h to the back of the mice, did not result in any difference in the number of apoptotic cells between vehicle- and DMBA-treated MK2−/− mice, whereas elevated numbers of apoptotic cells were seen in vehicle-treated MK2−/− skin and DMBA-treated MK2−/− skin (Figure 4A and B). No difference in the number of TUNEL-positive cells between TNF-α−/− and wild-type mice was found.

Increased stabilization of p53 in MK2−/−-deficient mice
Because the tumor suppressor protein p53 is known to play a key role in the induction of apoptosis, we next asked whether the increased apoptosis observed in MK2−/− mice was mediated through a regulation of p53. Western blotting analysis revealed that one application of DMBA for 24 h significantly increased the protein level of p53 in wild-type, MK2−/− and TNF-α−/− mice compared with vehicle-treated wild-type mice (P = 0.0004, P = 0.0003 and P = 0.0002, respectively) (Figure 5A). Interestingly, when DMBA treatment was followed by TPA treatment 6 h later, the level of p53 in the MK2−/− mice was even further increased, whereas the level of p53 was reduced in wild-type and TNF-α−/− mice, indicating that TPA caused a degradation of the p53 protein level in TNF-α−/− and wild-type mice (Figure 5A). No differences in the DMBA/TPA-induced p53 mRNA expression between the three groups of mice were seen (supplementary Figure 2A is available at Carcinogenesis Online).

The stability of p53 has previously been demonstrated to be regulated by Mdm2 (24,26). Furthermore, another study demonstrated that MK2 can phosphorylate Mdm2 leading to a reduction in p53 stability (27). To characterize the effect of TPA on the p38 MAPK/MK2/Mdm2-signaling pathway, studies were performed using cultured mouse keratinocytes. Stimulation of cultured wild-type keratinocytes with TPA caused a decrease in phospho-p38 MAPK (Figure 5B). Stimulation of cultured wild-type keratinocytes with TPA rapidly activated the p38 MAPK-signaling pathway as demonstrated by increased phosphorylation of p38 MAPK, MK2, and Mdm2 10 min after stimulation (Figure 5B). Knowing that TPA is a potent activator of the p38 MAPK/MK2/Mdm2-signaling pathway, we then stimulated cultured wild-type, MK2−/− and TNF-α−/− keratinocytes with TPA for 0, 10 and 30 min. The phosphorylation status of p38 MAPK between the three groups of mice keratinocytes after TPA stimulation was similar. In addition, no differences in the TPA-induced phosphorylation of MK2 between wild-type and TNF-α−/− keratinocytes were seen (Figure 5C). Interestingly, the level of phosphorylated Mdm2 was significantly lower in MK2−/− keratinocytes stimulated with TPA as compared with wild-type and TNF-α−/− keratinocytes (10 min: P = 0.024 and 30 min: P = 0.011) (Figure 5C). These findings are consistent with the decreased p53

Fig. 4. Response of MK2−/− and TNF-α−/− mice to DMBA/TPA-induced apoptosis. (A-C) The number of apoptotic cells per square millimeter epidermis was measured in different experimental settings. (A) Six mice in each group were treated with DMBA (25 μg) or vehicle followed 1 week later by four applications of TPA (4 μg) or vehicle over 7 days. (B) Five mice in each group were treated with one application of DMBA (25 μg) or vehicle for 24 h. For assessing apoptotic cells, 4 mm punch biopsies were taken and embedded in paraffin. Skin sections were then stained by the TUNEL method. Results are shown as the number of apoptotic cells per square millimeter epidermis (±SE). *P < 0.01 compared with wild-type mice. (C) Staining of apoptotic cells (TUNEL; green) and cell nuclei (4′,6-diamidino-2-phenylindole; blue) in skin of MK2−/− mice treated with DMBA/TPA. (D) Five mice in each group were treated with DMBA (25 μg) or vehicle followed 1 week later by four applications of TPA (4 μg) or vehicle over 7 days. Twenty-four hours after the last TPA application, whole cell extracts were isolated from skin biopsies taken from the back of the mice and analyzed by western blotting using antibodies directed against cleaved caspase-3 and β-actin. Scale bar, 25 μm; arrows indicate TUNEL-positive cells.
level after TPA treatment in wild-type and TNF-α-deficient mice but increased p53 protein level in MK2-deficient mice (Figure 5A). A reduced Mdm2 phosphorylation level in MK2−/− mice may lead to increased p53 protein levels due to reduced degradation. No differences in the DMBA/TPA-induced Mdm2 mRNA expression between the three groups of mice were observed (supplementary Figure 2B is available at Carcinogenesis Online).

These findings strongly suggest that increased p53 stability was responsible for the increased apoptosis in DMBA/TPA-treated MK2−/− cells, although a parallel pro-apoptotic pathway could also be involved. To prove that the MK2/Mdm2/p53-signaling pathway was responsible for the increased apoptosis, MK2−/− keratinocytes were cultured in the presence or absence of p53 siRNA. MK2−/− keratinocytes were transfected with control siRNA or p53 siRNA or...

Fig. 5. Loss of MK2 enhances the stabilization of p53. (A) Groups of mice (n = 5) were treated with one application of DMBA (25 μg) for 24 h or with DMBA followed 24 h later with one application of TPA (4 μg) for 6 h. Nuclear extracts were isolated from skin biopsies taken from the back of the mice and analyzed by western blotting using antibodies directed against p53 and Lamin B. (B and C) Cultures of keratinocytes from wild-type mice, MK2−/− mice and TNF-α−/− mice were stimulated with TPA (100 nM) for the indicated time points. Protein extracts were isolated and western blotting analysis used to measure the phosphorylated levels of p38 MAPK, MK2 and Mdm2. Representative gels of at least three different experiments are shown; n.s., non-specific. (D–F) Cultured MK2−/− keratinocytes were transfected with specific target siRNA (10 nM), control siRNA (10 nM) or transfection reagent alone (mock). (D) Transfection of MK2−/− keratinocytes with p53 siRNA, p65 siRNA, c-Jun siRNA or STAT3 siRNA led to a pronounced inhibition of p53, p65, c-Jun and STAT3 protein production as analyzed by western blotting. (E) Forty-eight hours after transfection, the keratinocytes were treated with DMBA (1 μM) followed by TPA (100 nM) treatment 6 h later. Twenty-four hours after DMBA treatment, the level of apoptosis was measured using the Cell Death Detection ELISAPLUS assay. Apoptosis [arbitrary units (a.u.)] is shown as mean (±SE) from three separate experiments. (F) MK2−/− keratinocytes were treated as in (E) before the activity of caspase-3 and -7 was analyzed by a Caspase-Glo® 3/7 assay kit. Caspase-3/caspase-7 activity (a.u.) is shown as mean (±SE) from three separate experiments. 'P < 0.05 compared with vehicle-treated keratinocytes. **P < 0.05 compared with keratinocytes transfected with non-specific control siRNA and treated with DMBA/TPA.
as a control, given transfection reagent only (mock). Gene silencing efficiency was verified both with western blotting (Figure 5D) and quantitative PCR (data not shown). Treatment of keratinocytes with DMBA/TPA significantly (P = 0.0002) increased the level of apoptosis compared with vehicle-treated cells (Figure 5E). Interestingly, when MK2−/− keratinocytes were transfected with p53 siRNA before DMBA/TPA treatment, the level of apoptosis was significantly (P = 0.0017) reduced compared with keratinocytes transfected with control siRNA and treated with DMBA/TPA (Figure 5E). The DMBA/TPA-induced apoptosis observed in MK2−/− keratinocytes was paralleled by an increased activity of caspase-3 and -7 (Figure 5F). Moreover, silencing of p53 prior to DMBA/TPA treatment significantly (P = 0.007) reduced caspase-3/caspase-7 activity compared with control siRNA-transfected cells (Figure 5F). We also analyzed the role of nuclear factor-kappaB, activator protein 1 and STAT3 on DMBA/TPA-induced apoptosis in MK2-deficient keratinocytes. Cells were transfected with p65 siRNA, c-Jun siRNA or STAT3 siRNA and gene-silencing efficiency was verified both with western blotting (Figure 5D) and quantitative PCR (data not shown). In contrast to p53 gene silencing, knockdown of p65, c-Jun or STAT3 in MK2-deficient keratinocytes did not influence on the DMBA/TPA-induced apoptosis (Figure 5E).

Together these results demonstrate that p53, but not nuclear factor-kappaB, activator protein 1 or STAT3, plays a key role in the DMBA/TPA-induced apoptosis observed in MK2−/− mice.

Discussion

Non-melanoma skin cancer (NMSC) is one of the most common human cancers and the incidence of NMSC is continuously rising (31). The increased incidence of NMSC has brought much attention to the process by which these tumors develop and how they can be prevented. A thorough understanding of the underlying molecular mechanisms leading to the development of NMSC after DNA damage is therefore pivotal.

Using MK2-deficient mice, we here demonstrate for the first time that MK2 is required for the development of skin tumors and is essential for maintaining the survival of DNA-damaged cells. MK2-deficiency made keratinocytes more sensitive to carcinogen-induced apoptosis through an impaired phosphorylation of Mdm2 and a subsequent increased stabilization of p53.

The development of cancer is strongly associated with inflammation. TNF-α is a proinflammatory cytokine that is mobilized during acute inflammation and known to mediate cancer development. Previous studies have demonstrated that mice deficient for either TNF-α or the TNF-α receptors show reduced susceptibility to chemically induced skin tumors (11,32). We found that the inhibition of the TPA-induced inflammatory response in MK2−/− mice was equal to that seen in TNF-α−/− mice when measured by the expression levels of the proinflammatory cytokines IL-1β and IL-6. Furthermore, there was no increased TNF-α protein expression after treatment with DMBA/TPA in MK2−/− mice compared with wild-type mice, where an increase was detected 24 h after the last TPA treatment. This is consistent with the well-documented role of MK2 in the regulation of TNF-α expression (13–15,33). The TPA-induced neutrophil cell infiltration to the skin was found to be significantly lower in TNF-α−/− mice compared with wild-type mice, which is in agreement with previous results (11). Moreover, we demonstrated that the skin infiltration of neutrophils in TNF-α−/− mice was significantly decreased compared with MK2−/− mice. Taken together, these data demonstrate that the inhibition of the TPA-induced inflammatory response is more pronounced in the TNF-α−/− mice compared with MK2−/− mice.

MK2−/− mice developed significantly fewer tumors compared with TNF-α−/− mice, indicating that the impaired inflammatory response and the decreased TNF-α expression seen in MK2−/− mice compared with wild-type mice cannot alone explain the lack of tumor development seen in MK2−/− mice in this carcinogenesis model.

We demonstrated increased numbers of apoptotic cells in the epidermis of MK2−/− mice compared with both wild-type and TNF-α−/− mice, demonstrating an inhibitory role of MK2 in the induction of apoptosis in the early stages of tumor promotion. The p53 protein is a key mediator of the DNA damage response and plays a critical role in the induction of apoptosis (34–36). In the present study, mice deficient in MK2 showed enhanced protein levels of p53 after DMBA/TPA treatment compared with wild-type and TNF-α−/− mice. In contrast, no regulation of the p53 mRNA expression was found between the three groups of mice, demonstrating that p53 is regulated at a posttranscriptional level. Moreover, in cultured MK2−/− keratinocytes transfected with p53 siRNA, the DMBA/TPA-induced apoptosis was significantly decreased, demonstrating the essential role of p53 in the induction of apoptosis in this model. Mdm2 has been described to act as a posttranscriptional regulator of p53, inactivating p53 by both repressing its transcriptional activity and augmenting degradation of p53 by the proteasome, thus downregulating p53 protein levels (37,38). MK2 appears to play a role in the posttranscriptional regulation of p53, as it was recently demonstrated that MK2 is able to phosphorylate Mdm2. In addition, cells deficient for MK2 showed reduced Mdm2 phosphorylation and elevated levels of p53 (27). Similar to these data, we demonstrated that the TPA-induced phosphorylation of Mdm2 in MK2-deficient mouse keratinocytes was significantly reduced compared with wild-type and TNF-α−/− mouse keratinocytes, whereas the phosphorylation status of p38 MAPK was not altered between the three groups of keratinocytes. Our data strongly suggest that in the early stages of tumor promotion, the inflammatory response inhibits apoptosis through an activation of the p38 MAPK/MK2-signaling pathway.

Besides of playing a critical role in the early stages of tumor initiation/promotion as demonstrated in this study, MK2 was recently shown to be essential in mediating cell death. This was demonstrated in an experimental model resembling the late stage of tumor promotion using p53-deficient cells (39). Reinhardt et al demonstrated that tumor cells lacking functional p53 survive DNA-damaging chemotherapeutic drugs through a p38 MAPK/MK2-dependent mechanism. In contrast, in p53-proficient cells, signaling through this pathway was dispensable for survival after DNA damage. These data together with our current results demonstrate that MK2 acts by different mechanisms to regulate the survival of cells in response to DNA damage, depending on the distinct stage in the carcinogenesis process.

Taken together, it appears that in skin carcinogenesis, MK2 acts as a double-edged sword regulating the inflammatory response through regulation of proinflammatory cytokine expression as well as regulating apoptosis through the p53-signaling pathway. The role and the importance of these findings seem to vary depending on the phases of the carcinogenesis process. Our results demonstrate that in the early stages of tumor initiation/promotion, loss of MK2 was dispensable for survival after DNA damage. These data together with our current results demonstrate that MK2 leads to decreased inflammatory response and increased p53 stabilization, resulting in increased numbers of DNA-damaged cells undergoing apoptosis. MK2 inhibitors may, therefore, be potential anticancer agents, inhibiting the early stages of carcinoma development.

Supplementary material

Supplementary data can be found at http://carcin.oxfordjournals.org/

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