Co-treatment with deoxycholic acid and azoxymethane accelerates secretion of HMGB1 in IEC6 intestinal epithelial cells

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Abstract

Objectives: High-mobility group box 1 (HMGB1) is a nuclear protein that acts as a ligand of the receptor for advanced glycation end products (RAGE) and its expression enhances progression of cancer. However, the mechanism underlying HMGB1 secretion is still unclear. In this study, we examined the effect of deoxycholic acid (DCA), a promoter of colon carcinogenesis, on HMGB1 secretion.

Materials and Methods: We used an *in vitro* transformation model comprised of IEC6 intestinal epithelial cells treated with azoxymethane (AOM) and/or DCA. HMGB1 expression and secretion were examined by Western and Northern blot analyses, and ELISA. Intracellular translocation of HMGB1 was examined by protein fractionation.

Results: AOM + DCA-treated IEC6 cells showed upregulation of HMGB1 mRNA expression and increased level of HMGB1 protein in culture medium, but decreased level of HMGB1 protein in the nucleus. AOM + DCA treatment increased level of histone H4 acetylation, which induced translocation of HMGB1 from the nucleus to the cytoplasm and increased HMGB1 secretion. Leptomycin B inhibited extranuclear translocation and secretion of the HMGB1 protein.

Conclusion: These findings suggest that DCA affects intracellular localization and secretion of HMGB1.

Introduction

High-mobility group box 1 (HMGB1) is a chromatin protein involved in processes such as gene transcription, DNA repair, cell differentiation, p53 function, and neural

development (1–4). Nuclear HMGB1 enhances expression of metastasis-related melanoma inhibitory activity in cooperation with nuclear factor-kappaB (5,6). HMGB1 knockout is lethal to mouse neonates, resulting from insufficient transcriptional activity of glucocorticoid receptor and subsequent inability in glycogen usage (7). HMGB1, also known as amphoterin, is involved in acceleration of cell motility. HMGB1 is closely associated with organ development; it plays an important role in increased rate of neurite outgrowth in the central nervous system (8,9).

Recently, HMGB1 has been recognized as a cytokine that participates in cancer progression and inflammatory diseases (10–13). Its release into the extracellular milieu is associated with endotoxin-induced organ injury, macrophage-associated inflammation, and cancer progression (14,15). We have reported that co-expression of the receptor for advanced glycation end products (RAGE) and its ligand HMGB1 is closely associated with cell proliferation, migration, and invasion. It is also associated with cancer progression and metastasis in gastric, colon, prostate, and oral cancers (6,16–22). HMGB1 induces apoptosis of macrophages, which is associated with colon cancer metastasis (19,21,23). Thus, secretion of HMGB1 plays a pivotal role in progression of various cancers. Because our previous studies have shown that co-expression of HMGB1 and RAGE is associated with adenoma-carcinoma progression in colorectal carcinogenesis (20), we sought to examine significance of HMGB1 expression in earlier stages of colon carcinogenesis, in the present study.

Although HMGB1 participates in diverse physiological and pathological processes as a cytokine, the mechanism of HMGB1 secretion remains to be elucidated. During inflammatory diseases, such as rheumatoid arthritis and sepsis, macrophages secrete HMGB1 (24–26). Lipopolysaccharide (endotoxin) is a strong stimulant of HMGB1 secretion by macrophages (27). In monocytes, HMGB1 is hyperacetylated prior to secretion, which is induced by stimuli triggering lysosome exocytosis (28,29). HMGB1 secretion is triggered by lysophosphatidylcholine, which is generated by monocytes present at a site of inflammation

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(29), whereas HMGB1 secretion from cancer cells is stimulated by growth factors and cytokines. HMGB1 mRNA expression and protein secretion are increased by transforming growth factor alpha and interleukin 15, which are frequently expressed in colon cancer cells (21,30).

In this study, we used azoxymethane (AOM) as a mutagen for the *in vitro* carcinogenesis model. AOM is converted to methylazoxymethanol by the enzyme CYP2E1 in the liver and CYP2E1 is expressed in IEC6 cells (33). Methylazoxymethanol promotes alkylation of DNA bases to generate point mutations (31,32). Thus, AOM plays a genotoxic role in this *in vitro* carcinogenesis model. Thus, the *in vitro* carcinogenesis model we used in this study comprised of IEC6 cells that were continuously treated with AOM for 60 weeks.

Deoxycholic acid (DCA) is a well-known promoter of colon carcinogenesis and acts as a transcription mediator, an epigenetic modulator, and an apoptosis inducer (34–39). In this report, we suggest that histone acetylation level that is altered by DCA enhances extranuclear translocation and secretion of HMGB1 during the transformation of intestinal epithelial cells.

Materials and methods

Cell culture

A rat intestinal epithelium cell line, IEC6 cells (ATCC accession CRL-1592), was purchased from Dainihon Pharmaceutical (Tokyo, Japan); it was established from rat small intestinal epithelium, but the genetic background is not well elucidated (40,41). Cells were routinely maintained in RPMI-1640 containing 10% foetal bovine serum (Sigma, St. Louis, MO, USA) under conditions of 5% CO₂ in air at 37 °C.

Pretreatment of IEC6 cells

IEC6 cells were pretreated with or without 100 µg/ml of DCA (Wako Pure Chemical Industries Ltd, Osaka, Japan) or 20 ng/ml of AOM (Sigma) with 100 µg/ml of DCA, for 60 weeks, with medium exchanges and additions of AOM or DCA every 7 days. Thereafter, cells of each group were washed twice in phosphate-buffered saline (PBS), harvested, and then seeded on glass slides. This *in vitro* carcinogenesis model needs continuous treatment with AOM on the long term as treated cells are needed to be split periodically to maintain culture.

Immunocytochemistry

The cultured cells were rinsed twice with PBS and split following brief trypsin treatment, then fixed in 10%

buffered formalin and resuspended in 1% agarose–PBS solution at 37 °C. Gels containing cells were processed to paraffin embedding and section cutting. Antigen retrieval was performed with microwave pretreatment in citrate buffer pH 6.0 (Dako Corp., Carpinteria, CA, USA) for 20 min, and immunostaining was carried out using the immunoperoxidase technique. After endogenous peroxidase blocking with 3% H₂O₂–methanol for 15 min and specimens were rinsed with PBS. Anti-HMGB1 antibody or anti-acetylated histone H4 (Upstate Biotechnology Inc., Lake Placid, NY, USA) were used respectively at 1.0 µg/ml, as primary antibodies and incubated at room temperature for 2 h. Specimens were rinsed with PBS and incubated at room temperature for 1 h with secondary antibody conjugated with peroxidase or fluorescein isothiocyanate diluted to 0.5 µg/ml (anti-goat immunoglobulin G, MBL Co. Ltd., Nagoya, Japan). All specimens were then rinsed in PBS again and were colour-developed with diaminobenzidine solution (Dako). After washing in water, specimens were counterstained with Meyer's haematoxylin (Sigma). Immunostaining of all specimens was performed to ensure the same conditions of antibody reaction and diaminobenzidine exposure.

In situ hybridization

In situ hybridization was performed as described previously (17,18), sequence of the microprobe for HMGB1 being 5'-CCC AGT TTC TTC GCA ACA TCA C-3' (referred to as GenBank accession no. X12597) (17), and the probe conjugated to biotin at the 5'-end (Sigma Genosys, Ishikari, Japan). Non-counterstained *in situ* hybridization slides were examined by densitometric scanning with computer-capturing image analysis (NIH Image software, National Institutes of Health, Bethesda, MD, USA). Images of specimens were stored on a computer and processed. Briefly, 1000 cells were identified from images, and hybridization signals were scanned as greyscale images using the NIH Image software. Mean signal density and mean nuclear area of the 1000 cells were calculated.

Reverse transcriptase–polymerase chain reaction

RNA was extracted from cultured cells using the RNeasy Mini Kit (Qiagen Genomics Inc., Bothell, WA). RNA (1 µg) was converted to cDNA totally using First-Strand cDNA Synthesis Kit (Amersham Biosciences Corp., Piscataway, NJ, USA). Polymerase chain reactions were performed using AmpliTaq Gold Kit, following the manufacturer's instructions (Applied Biosynthesis, Foster City, CA, USA). Primer pair of HMGB1 was sense: 5'-ATA TGG CAA AAG CGG ACA AG-3', anti-sense: 5'-GCA ACA

TCA CCA ATG GAC AG-3' (GenBank accession no. NM002128), and that of β -actin was sense: 5'-CAA GAG ATG GCC ACG GCT GCT-3', anti-sense: 5'-TCC TTC TGC ATC CTG TCG GCA-3' (GenBank accession no. NM001101). All primers were synthesized by Sigma Genosys (Ishikari, Japan).

Enzyme-linked immunosorbent assay for HMGB1

Enzyme-linked immunosorbent assay (ELISA) was performed to measure protein concentrations of HMGB1 (Shino-Test, Tokyo, Japan), the assay being performed according to the source instructions, in triplicate. Conditioned medium (200 μ l) was filtered using a 0.2- μ m filter (Becton-Dickinson Labware, Bedford, MA, USA), or serum (200 μ l) was used for ELISA. The experiment was repeated three times.

Treatment with trichostatin A and leptomycin B

Stock solution of trichostatin A (TSA, Wako Pure Chemical Industries Ltd, 10 ng/ μ l) and leptomycin B (LMB, Sigma, 5 ng/ μ l) were prepared. TSA and LMB stock solutions were added directly to culture medium to final concentrations of 50 ng/ml and 20 ng/ml, respectively.

Immunoblot analysis

Harvested cells were resuspended in 500 μ l of STKM buffer (50 mM Tris-HCl pH 7.6, 25 mM KCl, 5 mM MgCl₂, 0.25 M sucrose, 20 μ g/ml leupeptin, 1% PMSF) and were homogenized by 150 strokes in Dounce pestles. After 1000 g, 5-min centrifugation, the supernatant was centrifuged at 40 000 g (1 h, 4 °C), dividing pellet (membrane fraction) and supernatant (cytosol fraction), which were resuspended with 1 $\times$ lysis buffer (50 mM Tris pH 7.4, 5 mM EDTA, 1 mM EGTA, 1% NP-40, 20 μ g/ml leupeptin, 1% PMSF). The pellet was resuspended in hypertonic buffer (10 mM HEPES pH 7.9, 5 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT), incubated for 5 min on ice, then the same amount of 2 $\times$ TKMS buffer was added. After centrifugation at 40 000 g (1 h, 4 °C), the supernatant (Nuclear fraction) was saved. Whole-cell lysates were prepared as described previously (42). Lysates and fractions (20 μ g) were used for immunoblot analysis in 12.5% sodium dodecyl sulphate–polyacrylamide gels followed by electrotransfer to nitrocellulose filters. Filters were incubated with anti-HMGB1 antibody or anti-acetylated histone H4 (Upstate Biotechnology), and then with peroxidase-conjugated immunoglobulin G antibody (MBL, Nagoya, Japan). Anti- γ -tubulin antibody was used to assess levels of protein loaded per lane (Oncogene Research Products, Cambridge, MA, USA). The immune complex was visualized using

enhanced chemiluminescence–Western blot detection system (Amersham, Aylesbury, UK). For semiquantitative analysis, specific signals of immunoblotted membranes were digitized and quantified using NIH Image computer software (National Institutes of Health).

Animal and diets

The study was approved by the Committee for Animal Experimentation of Nara Medical University. Five-week-old male F344 rats (Japan SLC Inc., Shizuoka, Japan) were acclimatized for 1 week and then randomized into experimental and control groups. Four animals were housed in each wide cage. The holding room was maintained at 23 °C and 50% humidity, with 12-h light/dark cycle. Control diet (CE-2) was purchased from Clea Inc. (Tokyo, Japan). At 6 weeks of age, five rats were fed CE-2 control diet and water. The other five rats were fed CE-2 control diet and DCA solution (1% DCA in filtrated water). Rats were killed under ether anaesthesia 2 weeks after drinking DCA solution. Cardiac blood was collected and serum was saved after coagulation. After the colon was removed, colonic mucosa was scraped off, frozen in liquid nitrogen, and stored at –80 °C for further analysis.

Statistical analyses

Statistical significance was analysis of variance, and unpaired Mann–Whitney test using Statview 4.51 software (Abacus Concepts Inc., Berkeley, CA, USA). Statistical significance was defined as two-sided *P*-value of less than 0.05.

Results

Expression and secretion of HMGB1 in IEC6 cells

We examined HMGB1 protein production in IEC6 cells by using immunohistochemistry (Fig. 1a). Immunoreactivity was observed at the nucleus of untreated IEC6 cells. Cells treated with DCA (100 μ g/ml) or AOM (20 ng/ml) exhibited obvious staining of HMGB1 in nuclei and cytoplasm, but most IEC6 cells treated with DCA + AOM demonstrated no nuclear HMGB1 staining. The number of nuclei positive for HMGB1 in IEC6 cells was decreased in both DCA and DCA + AOM treatment (*P* = 0.0058 and *P* = 0.0003, respectively) (Fig. 1b); decrease in level of HMGB1 protein in cells treated with DCA + AOM was confirmed by Western blot analysis (Fig. 1c). Increase in levels HMGB1 mRNA due to treatment with AOM, DCA, and AOM + DCA was confirmed by *in situ* hybridization (Fig. 1d) and reverse transcriptase–polymerase chain reaction (RT-PCR) (Fig. 1e). The increase was substantial in the

Figure 1. Effect of deoxycholic acid (DCA) and/or azoxymethane (AOM) on HMGB1 levels in IEC6 cells. (a) Immunohistochemistry of HMGB1 in IEC6 cells. (b) Nuclear HMGB1-positivity per 1000 cells were counted. Error bar: standard deviation (SD) (c) Western blot analysis of HMGB1 expression in IEC6 cells. Tubulin served as a loading control. (d) *In situ* hybridization of HMGB1 in IEC6 cells. Cells were not counter-stained. (e) Reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of HMGB1 mRNA expression in IEC6 cells. β -actin served as an internal control. (f) HMGB1 mRNA expression was semiquantitated by densitometric scanning of RT-PCR signals. In order to measure expression level of HMGB1 mRNA, signal intensities of HMGB1 were compared with those of β -actin. Mean $\pm$ SD, calculated from three independent experiments. (g) HMGB1 concentration in the culture media of IEC6 cells.

case of the AOM + DCA treatment. HMGB1 expression level was 1.4, 1.2, and 6.3 times higher in cells treated with AOM, DCA, and DCA + AOM, respectively, than in untreated cells (Fig. 1f). In particular, total HMGB1 expression in DCA + AOM-treated cells was 215% that

of untreated cells ($P = 0.0005$). Next, we measured levels of the HMGB1 protein in culture medium by using ELISA. The level of secreted HMGB1 protein increased to 22 $\mu\text{g/ml}$ and 27 $\mu\text{g/ml}$ in cells treated with AOM and DCA, respectively. Furthermore, HMGB1 secretion in DCA +

Figure 2. Acetylation levels of histone H4 in IEC6 cells. (a) Immunohistochemistry of acetylated histone H4 (AcH4) in IEC6 cells. (b) AcH4-positive cells per 1000 cells were counted. Mean $\pm$ standard deviation, calculated from three independent experiments. (c) Western blot analysis of AcH4 in IEC6 cells. Tubulin served as a loading control.

AOM-treated cells was three times greater than that in untreated ones (16 $\mu\text{g}/\text{ml}$) ($P < 0.0001$) (Fig. 1g). Thus, co-treatment with AOM and DCA induced upregulation of HMGB1 mRNA expression and increase in HMGB1 protein level in the culture medium, but decrease in nuclear HMGB1 protein level.

Effect of DCA on histone H4 acetylation

Histone acetylation level in these IEC6 cells was determined by immunohistochemistry. Level of acetylated histone H4 was higher in samples treated with DCA + AOM than in control samples (Fig. 2a). Level of acetylated histone H4 following treatment with DCA or DCA + AOM increased by 1.8 and 2.5 times, respectively compared to untreated cells ($P = 0.0060$ and $P = 0.0031$, respectively) (Fig. 2b). Levels of acetylated histone H4 were also

Figure 3. Effect of trichostatin A (TSA) and leptomycin B (LMB) on subcellular localization and secretion of HMGB1 in IEC6 cells. (a) Fluorescent immunocytochemistry of HMGB1. TSA treatment decreased nuclear HMGB1 protein in IEC6 cells. (b) Alteration of intracellular localization of HMGB1 in TSA- or LMB-treated IEC6 cells. Mem, membrane fraction; Cyt, cytosol fraction; Nuc, nuclear fraction. (c) Effect of LMB on secretion of HMGB1. HMGB1 concentration in culture medium of IEC6 cells was determined using ELISA. Mean $\pm$ standard deviation, calculated from three independent experiments.

examined by Western blot analysis (Fig. 2c). IEC6 cells treated with AOM + DCA had an increase in acetylated histone H4 level.

Effect of TSA and LMB on HMGB1 translocation and secretion

In order to determine the effect of histone H4 acetylation on extranuclear translocation of HMGB1, we treated our IEC6 cells with TSA, a histone deacetylase inhibitor. Treatment with TSA decreased HMGB1 secretion in the nuclear fraction and increased it in the membrane fractions of the cells (Fig. 3a,b). In contrast, co-treatment with TSA and LMB, a nuclear export inhibitor, showed inhibition of HMGB1 translocation from the nuclear fraction to the cytosolic fraction, where HMGB1 level was lower in

Figure 4. Effect of deoxycholic acid (DCA) and/or azoxymethane (AOM) on HMGB1 levels and histone H4 acetylation in rat colonic mucosa.

(a) Five F344 Fisher rats in each group were injected with or without AOM (15 mg/kg body weight subcutaneously) and/or DCA (1% in drinking water). (b) Serum HMGB1 levels in each group. HMGB1 concentration was measured using ELISA. Mean $\pm$ standard deviation, calculated from five samples. HMGB1 concentrations in Groups A, B, and C were significantly different from the concentration in Group D, as indicated by the *P* values. (c) HMGB1 mRNA expression and levels of nuclear HMGB1 protein and acetylated histone H4. mRNA levels were detected using reverse transcriptase–polymerase chain reaction, and protein levels were determined by Western blot analysis with nuclear extracts. β -actin and Coomassie blue (CB) served as loading controls.

LMB-treated cells than in untreated cells. Additionally, effects of TSA and LMB on HMGB1 secretion were examined (Fig. 3c). HMGB1 secretion in TSA-treated cells increased so that it was 287% of that in untreated cells. However, LMB treatment decreased TSA-induced HMGB1 secretion so that it was 14% that in TSA-treated cells ($P = 0.0003$) and 39% that in controls ($P = 0.0051$). Thus, histone acetylation increased extranuclear translocation of HMGB1 *via* the nuclear export system.

Effect of DCA on HMGB1 secretion in rat colonic mucosa

Male Fisher 344 rats were treated with AOM and/or DCA, as shown in Fig. 4a. Two weeks after treatment, serum HMGB1 levels were determined (Fig. 4b). Group B (AOM + DCA-treated rats) showed evident increase in serum HMGB1 levels as compared to such levels in Group D (control rats) ($P = 0.0005$). Group A (AOM-treated rats) and Group C (DCA-treated rats) also showed higher serum HMGB1 levels than the level shown by Group D ($P = 0.0036$ and $P = 0.0083$, respectively). Compared to Group D, Group B showed pronounced increase in HMGB1 mRNA level, whereas there was marked reduction in nuclear HMGB1 protein level. Groups A and C also showed modest alteration in levels of HMGB1 mRNA and nuclear HMGB1. Moreover, among all four groups, levels of acetylated histone H4 were highest in Group B, followed by Groups A and C.

Acetylated histone H4 levels correlated well with serum HMGB1 concentration. Thus, co-treatment with AOM and DCA induced HMGB1 translocation and secretion, which was linked to histone H4 acetylation in the animal models.

Discussion

DCA, a major component of secondary bile acids, has been reported to act as an endogenous colon tumour promoter. For example, rats treated with AOM and fed a diet supplemented with DCA developed a higher number of tumours than those treated with AOM alone (35,37,39). Other reports have confirmed that DCA directly stimulates proliferation of human colonic mucosa and human colon cancer cells, in culture (34,36). However, the relationship between HMGB1 and DCA has not been studied previously.

We used AOM as a mutagen for the *in vitro* carcinogenesis model and then evaluated the effect of DCA on HMGB1 secretion in the IEC6 cells (43). We studied intracellular localization of HMGB1 protein; AOM + DCA treatment upregulated HMGB1 mRNA expression and decreased level of nuclear HMGB1. These results suggested that AOM + DCA treatment caused extranuclear translocation of HMGB1. Additionally, these results were supported by findings that DCA + AOM treatment increased HMGB1 secretion as much as three times that measured from

untreated cells. In the AOM-induced rat colon carcinogenesis model, we confirmed that DCA increased HMGB1 secretion in colonic mucosa. HMGB1 provides variable pro-carcinogenic effects through RAGE, which has roles in growth, motility and survival of cancer cells, as well as in angiogenesis (25). Nuclear HMGB1 has a role in DNA repair (44); thus, it provides a potential anti-carcinogenic effect. We demonstrated that AOM + DCA treatment induced extranuclear localization, and subsequent decrease of nuclear HMGB1. This alteration could impair DNA repair. Therefore, these findings suggest that one of the mechanisms of DCA as a carcinogenic promoter is linked to induction of HMGB1 secretion.

Our data showed that AOM and/or DCA treatment increased histone H4 acetylation; this increase was more pronounced in the case of the AOM + DCA treatment. Experiments with TSA and LMB confirmed our previous finding that acetylated histone H4 induces extranuclear translocation and secretion of HMGB1. Scaffidi *et al.* reported passive release of HMGB1 from degraded nuclei of necrotic cancer cells but not from hypo-acetylated apoptotic cells (45). We confirmed that concentrations of DCA and/or AOM used in the experiments did not affect viability of IEC6 cells. These findings suggest that loosening of chromatin structure by histone acetylation releases HMGB1 from chromatin; released HMGB1 is then transported to the cytoplasm by an active export system. Hyperacetylation-induced HMGB1 secretion was confirmed in the animal colon mucosa treated with AOM and/or DCA.

The mechanism of HMGB1 secretion is associated with atypical endolysosomal-like secretory pathway in human monocytes (29). In our study, treatment with TSA did not affect cytosolic HMGB1 secretion, suggesting the possibility of excess cytosolic HMGB1 secretion. In contrast, membrane HMGB1 secretion was increased by TSA treatment and decreased by LMB treatment. This finding is compatible with those of Bonaldi *et al.* that cytosolic HMGB1 is concentrated in secretory lysosomes (28). Thus, vesicular system-associated HMGB1 might be secreted from the cytoplasm. Enterocytes that are stimulated by inflammatory cytokines (tumour necrosis factor, interleukin 1 β , and interferon gamma) are reported to secrete HMGB1 in the exosome-associated form (46). In our study, HMGB1 was detected in the culture medium of IEC6 cells without solubilization; this suggests that HMGB1 that was secreted into the medium of the cells was present in solution and was not associated with membrane-bound vesicles. Inflammatory cytokines-stimulated enterocytes secrete HMGB1 at the apical portion of cells but not at the basolateral portion (46). In order to confirm polarity of HMGB1 secretion in AOM + DCA-treated IEC6 cells, cells were cultured as a monolayer on type IV collagen membrane in an insert.

In this system, HMGB1 was detected in both upper and lower chambers at the same concentration. Therefore, IEC6 cells transformed by AOM + DCA differed from the enterocytes in that the former secreted HMGB1 in a non-polarized manner.

In conclusion, we showed that DCA increases histone H4 acetylation in IEC6 cells and affects intracellular localization and secretion of HMGB1. DCA-induced HMGB1 secretion might be associated with colon carcinogenesis and disease progression. Serum HMGB1 level is high in hepatocellular carcinomas and non-Hodgkin lymphomas (47,48). Our data also showed that serum HMGB1 level increased in colon carcinogenesis. Thus, secreted HMGB1 might be a marker for cancer development and progression. Secreted HMGB1 is also an important target for cancer treatment. Further studies are required on neutralization of secreted HMGB1 and inhibition of HMGB1 secretion as a means to suppress cancer development and progression.

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References

- 1 Goodwin GH, Sanders C, Johns EW (1973) A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. *Eur. J. Biochem.* **38**, 14–19.
- 2 Pasheva EA, Ugrinova I, Spassovska N, Pashev IG (2002) The binding affinity of HMGI protein to DNA modified by cis-platin and its analogs correlates with their antitumor activity. *Int. J. Biochem. Cell Biol.* **34**, 87–92.
- 3 Stros M, Ozaki T, Bacikova A, Kageyama H, Nakagawara A (2002) HMGB1 and HMGB2 cell-specifically down-regulate the p53- and p73-dependent sequence-specific transactivation from the human Bax gene promoter. *J. Biol. Chem.* **277**, 7157–7164.
- 4 Ugrinova I, Pasheva EA, Armengaud J, Pashev IG (2001) *In vivo* acetylation of HMGI protein enhances its binding affinity to distorted DNA structures. *Biochemistry* **40**, 14655–14660.
- 5 Poser I, Golob M, Buettner R, Bosserhoff AK (2003) Upregulation of HMGI leads to melanoma inhibitory activity expression in malignant melanoma cells and contributes to their malignancy phenotype. *Mol. Cell. Biol.* **23**, 2991–2998.
- 6 Sasahira T, Kirita T, Oue N, Bhawal UK, Yamamoto K, Fujii K, Ohmori H, Luo Y, Yasui W, Bosserhoff AK, Kuniyasu H (2008) High mobility group box-1-inducible melanoma inhibitory activity is associated with nodal metastasis and lymphangiogenesis in oral squamous cell carcinoma. *Cancer Sci.* **99**, 1806–1812.
- 7 Calogero S, Grassi F, Aguzzi A, Voigtlander T, Ferrier P, Ferrari S, Bianchi ME (1999) The lack of chromosomal protein Hmg1 does not disrupt cell growth but causes lethal hypoglycaemia in newborn mice. *Nat. Genet.* **22**, 276–280.
- 8 Huttunen HJ, Kuja-Panula J, Sorci G, Agneletti AL, Donato R, Rauvala H (2000) Coregulation of neurite outgrowth and cell survival by amphotericin and S100 proteins through receptor for advanced

- glycation end products (RAGE) activation. *J. Biol. Chem.* **275**, 40096–40105.
- 9 Parkkinen J, Rauilo E, Merenmies J, Nolo R, Kajander EO, Baumann M, Rauvala H (1993) Amphoterin, the 30-kDa protein in a family of HMGI-type polypeptides. Enhanced expression in transformed cells, leading edge localization, and interactions with plasminogen activation. *J. Biol. Chem.* **268**, 19726–19738.
 - 10 Andersson U, Erlandsson-Harris H, Yang H, Tracey KJ (2002) HMGB1 as a DNA-binding cytokine. *J. Leukoc Biol.* **72**, 1084–1091.
 - 11 Kim JY, Park JS, Strassheim D, Douglas I, Diaz del Valle F, Asehnoune K, Mitra S, Kwak SH, Yamada S, Maruyama I, Ishizaka A, Abraham E (2005) HMGB1 contributes to the development of acute lung injury after hemorrhage. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **288**, L958–L965.
 - 12 Yang H, Wang H, Tracey KJ (2001) HMG-1 rediscovered as a cytokine. *Shock* **15**, 247–253.
 - 13 Yang H, Wang H, Czura CJ, Tracey KJ (2002) HMGB1 as a cytokine and therapeutic target. *J. Endotoxin Res.* **8**, 469–472.
 - 14 Tang D, Shi Y, Jang L, Wang K, Xiao W, Xiao X (2005) Heat shock response inhibits release of high mobility group box 1 protein induced by endotoxin in murine macrophages. *Shock* **23**, 434–440.
 - 15 Watanabe T, Kubota S, Nagaya M, Ozaki S, Nagafuchi H, Akashi K, Taira Y, Tsukikawa S, Oowada S, Nakano S (2005) The role of HMGB-1 on the development of necrosis during hepatic ischemia and hepatic ischemia/reperfusion injury in mice. *J. Surg. Res.* **124**, 59–66.
 - 16 Bhawal UK, Ozaki Y, Nishimura M, Sugiyama M, Sasahira T, Nomura Y, Sato F, Fujimoto K, Sasaki N, Ikeda M, Tsuji K, Kuniyasu H, Kato Y (2005) Association of expression of receptor for advanced glycation end products and invasive activity of oral squamous cell carcinoma. *Oncology* **69**, 246–255.
 - 17 Kuniyasu H, Oue N, Wakikawa A, Shigeishi H, Matsutani N, Kuraoka K, Ito R, Yokozaki H, Yasui W (2002) Expression of receptors for advanced glycation end-products (RAGE) is closely associated with the invasive and metastatic activity of gastric cancer. *J. Pathol.* **196**, 163–170.
 - 18 Kuniyasu H, Chihara Y, Takahashi T (2003) Co-expression of receptor for advanced glycation end products and the ligand amphoterin associates closely with metastasis of colorectal cancer. *Oncol. Rep.* **10**, 445–448.
 - 19 Kuniyasu H, Sasaki T, Sasahira T, Ohmori H, Takahashi T (2004) Depletion of tumor-infiltrating macrophages is associated with amphoterin expression in colon cancer. *Pathobiology* **71**, 129–136.
 - 20 Sasahira T, Akama Y, Fujii K, Kuniyasu H (2005) Expression of receptor for advanced glycation end products and HMGB1/amphoterin in colorectal adenomas. *Virchows Arch.* **446**, 411–415.
 - 21 Sasahira T, Sasaki T, Kuniyasu H (2005) Interleukin-15 and transforming growth factor alpha are associated with depletion of tumor-associated macrophages in colon cancer. *J. Exp. Clin. Cancer Res.* **24**, 69–74.
 - 22 Sasahira T, Kirita T, Bhawal UK, Yamamoto K, Kuniyasu H (2007) Receptor for advanced glycation end products (RAGE) is important in the prediction of recurrence in human oral squamous cell carcinoma. *Histopathology* **51**, 166–172.
 - 23 Kuniyasu H, Yano S, Sasaki T, Sasahira T, Sone S, Ohmori H (2005) Colon cancer cell-derived high mobility group 1/amphoterin induces growth inhibition and apoptosis in macrophages. *Am. J. Pathol.* **166**, 751–760.
 - 24 Andersson U, Erlandsson-Harris H (2004) HMGB1 is a potent trigger of arthritis. *J. Intern. Med.* **255**, 344–350.
 - 25 Huttunen HJ, Rauvala H (2004) Amphoterin as an extracellular regulator of cell motility: from discovery to disease. *J. Intern. Med.* **255**, 351–366.
 - 26 Wang H, Yang H, Tracey KJ (2004) Extracellular role of HMGB1 in inflammation and sepsis. *J. Intern. Med.* **255**, 320–331.
 - 27 Czura CJ, Wang H, Tracey KJ (2001) Dual roles for HMGB1: DNA binding and cytokine. *J. Endotoxin Res.* **7**, 315–321.
 - 28 Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, Bachi A, Rubartelli A, Agresti A, Bianchi ME (2003) Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. *EMBO J.* **22**, 5551–5560.
 - 29 Gardella S, Andrei C, Ferrera D, Lotti LV, Torrisi MR, Bianchi ME, Rubartelli A (2002) The nuclear protein HMGB1 is secreted by monocytes via a non-classical, vesicle-mediated secretory pathway. *EMBO Rep.* **3**, 995–1001.
 - 30 Kuniyasu H, Yasui W, Shinohara H, Yano S, Ellis LM, Wilson MR, Bucana CD, Rikita T, Tahara E, Fidler IJ (2000) Induction of angiogenesis by hyperplastic colonic mucosa adjacent to colon cancer. *Am. J. Pathol.* **157**, 1523–1535.
 - 31 Feinberg A, Zedeck MS (1980) Production of a highly reactive alkylating agent from the organospecific carcinogen methylazoxymethanol by alcohol dehydrogenase. *Cancer Res.* **40**, 4446–4450.
 - 32 Sohn OS, Ishizaki H, Yang CS, Fiala ES (1991) Metabolism of azoxymethane, methylazoxymethanol and N-nitrosodimethylamine by cytochrome P450IIE1. *Carcinogenesis* **12**, 127–131.
 - 33 Sasaki T, Yoshida K, Shimura H, Ichiba M, Sasahira T, Shimomoto T, Denda A, Kuniyasu H (2006b) Inhibitory effect of linoleic acid on transformation of IEC6 intestinal cells by *in vitro* azoxymethane treatment. *Int. J. Cancer* **118**, 593–599.
 - 34 Bartram HP, Scheppach W, Schmid H, Hofmann A, Dusel G, Richter F, Richter A, Kasper H (1993) Proliferation of human colonic mucosa as an intermediate biomarker of carcinogenesis: effects of butyrate, deoxycholate, calcium, ammonia, and pH. *Cancer Res.* **53**, 3283–3288.
 - 35 Narisawa T, Magadia NE, Weisburger JH, Wynder EL (1974) Promoting effect of bile acids on colon carcinogenesis after intra-rectal instillation of N-methyl-N'-nitro-N-nitrosoguanidine in rats. *J. Natl. Cancer Inst.* **53**, 1093–1097.
 - 36 Peiffer LP, Peters DJ, McGarrity TJ (1997) Differential effects of deoxycholic acid on proliferation of neoplastic and differentiated colonocytes *in vitro*. *Dig. Dis. Sci.* **42**, 2234–2240.
 - 37 Reddy BS, Watanabe K, Weisburger JH, Wynder EL (1977) Promoting effect of bile acids in colon carcinogenesis in germ-free and conventional F344 rats. *Cancer Res.* **37**, 3238–3242.
 - 38 Sasaki T, Sasahira T, Shimura H, Ikeda S, Kuniyasu H (2005) High concentration of deoxycholic acid abrogates *in vitro* transformation of IEC6 intestinal cells by azoxymethane. *J. Exp. Clin. Cancer Res.* **24**, 207–213.
 - 39 Sutherland LA, Bird RP (1994) The effect of chenodeoxycholic acid on the development of aberrant crypt foci in the rat colon. *Cancer Lett.* **76**, 101–107.
 - 40 Contes CN, Majumdar AP (1987) The effects of gastrin, epidermal growth factor, and somatostatin on DNA synthesis in a small intestinal crypt cell line (IEC-6). *Proc. Soc. Exp. Biol. Med.* **184**, 307–311.
 - 41 Quaroni A, Isselbacher KJ, Ruoslahti E (1978) Fibronectin synthesis by epithelial crypt cells of rat small intestine. *Proc. Natl. Acad. Sci. USA* **75**, 5548–5552.
 - 42 Kuniyasu H, Yasui W, Kitahara K, Naka K, Yokozaki H, Akama Y, Hamamoto T, Tahara E (1997) Growth inhibitory effect of interferon-beta is associated with the induction of cyclin-dependent kinase inhibitor p27Kip1 in a human gastric carcinoma cell line. *Cell Growth Differ.* **8**, 47–52.
 - 43 Sasaki T, Fujii K, Yoshida K, Shimura H, Sasahira T, Ohmori H, Kuniyasu H (2006) Peritoneal metastasis inhibition by linoleic acid with activation of PPAR γ in human gastrointestinal cancer cells. *Virchows Arch.* **448**, 422–427.

- 44 Lange SS, Mitchell DL, Vazquez KM (2008) High mobility group protein B1 enhances DNA repair and chromatin modification after DNA damage. *Proc. Natl. Acad. Sci. USA* **105**, 10320–10325.
- 45 Scaffidi P, Misteli T, Bianchi ME (2002) Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* **418**, 191–195.
- 46 Liu S, Stolz DB, Sappington PL, Macias CA, Killeen ME, Tenhunen JJ, Delude RL, Fink MP (2006) HMGB1 is secreted by immunostimulated enterocytes and contributes to cytomix-induced hyperpermeability of Caco-2 monolayers. *Am. J. Physiol. Cell Physiol.* **290**, C990–C999.
- 47 Cheng BQ, Jia CQ, Liu CT, Lu XF, Zhong N, Zhang ZL, Fan W, Li YQ (2008) Serum high mobility group box chromosomal protein 1 is associated with clinicopathologic features in patients with hepatocellular carcinoma. *Dig. Liver Dis.* **40**, 446–452.
- 48 Meyer A, Staratschek-Jox A, Springwald A, Wenk H, Wolf J, Wickenhauser C, Bullerdiek J (2008) Non-Hodgkin lymphoma expressing high levels of the danger-signalling protein HMGB1. *Leuk. Lymphoma.* **49**, 1184–1189.