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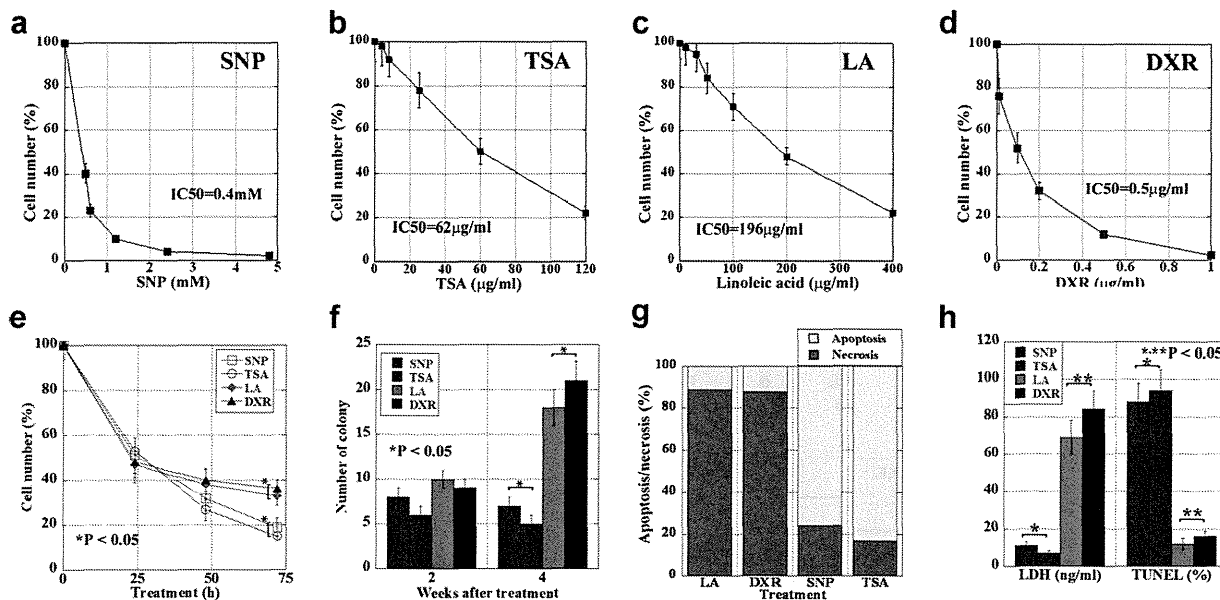

Fig. 1. Effect of cell death inducers on CT26 cell growth. (a–d) The growth inhibitory effect of sodium nitroprusside (SNP), trichostatin A (TSA), linoleic acid (LA) and doxorubicin (DXR) on CT26 cells was examined at various concentrations. Cell number was counted by an autocytometer. The 50% inhibitory concentration (IC50) after 24-h treatment is also indicated. (e) The time course of the cell number treated with SNP, TSA, LA and DXR at the IC50. (f) The colony forming assay after 48-h treatment of SNP, TSA, LA and DXR at the IC50. The colony numbers were counted at 2 and 3 weeks after treatment. (g) The apoptosis/necrosis ratio of dead cells was examined in CT26 cells treated with IC50 for 24 h. (h) To confirm the pro-apoptotic or pro-necrotic effects of SNP, TSA, LA and DXR, LDH concentration in the culture media and Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL)-positive cell percentage were examined in cells after 48-h treatment of the four agents. Error bars represent standard deviation (SD).

IC50 (Fig. 2a and b) and the half concentration of IC50 (Fig. 2c and d). CT26 cells treated with the medium in which DXR-treated cells were cultured showed growth enhancement in comparison with cells cultured in the control (PBS-treated) medium. In contrast, cells treated with the medium in which TSA-treated cells were cultured did not show growth enhancement. Treatment with DXR of the half concentration of IC50 showed less pronounced effect than that of IC50 DXR treatment ($P < 0.05$). These findings suggest that a specific humoral factor was responsible for the necrotic cell-associated cell growth. Because HMGB1 is a known necrosis sensor, the presence of HMGB1 was examined in the culture medium (CM) of CT26 cells treated with cell death inducers (Fig. 2e). Necrosis inducers (DXR and LA) yielded higher levels of HMGB1 in the CM in a dose-dependent manner in comparison with apoptosis inducers did (TSA and SNP) ($P < 0.0001$). Using the CM of 72-h treatment, CT26 cells treated with 50% DXR-CM and 70% DXR-CM showed $126 \pm 18\%$ and $122 \pm 15\%$ cell numbers, respectively. Oxidised HMGB1 lacks ligand activity.[26,27] The less proliferative effect of 72-h treated DXR-CM than that of 24-h treated DXR-CM might be resulted from increased oxidised HMGB1 portion (Fig. 2f). HMGB1 is reported to enhance cell survival by autophagy induction.[27,28] To examine the pro-autophagy effect of HMGB1, production of LC3 was detected by immunoblotting, which is one of autophagy machinery proteins. In Fig. 2g, necrosis-inducers increased LC3 levels; however, apoptosis-inducers did not.

3.3. Effect of cell death inducers on HMGB1 release in a mouse tumour model

CT26 subcutaneous tumours on the back of BALB/c mice were treated with DXR (5 and 10 μg/mouse) or TSA (0.6 and 1.2 mg/mouse) by intraperitoneal (i.p.) administration. DXR treatment produced necrotic areas; however, a few apoptotic cells were also observed in the tumour (Fig. 3a). In contrast, TSA treatment resulted in numerous TUNEL-positive apoptotic cells, but failed to produce necrotic areas. TSA treatment showed that the number of apoptotic cells was increased in a dose-dependent manner and was higher than that by DXR treatment (Fig. 3b, $P < 0.0001$). In contrast, DXR treatment showed that serum LDH was increased in a dose-dependent manner and was higher than that by TSA treatment (Fig. 3c, $P < 0.0001$). Furthermore, in the DXR-treated mice, the serum concentration of HMGB1 was increased in a dose-dependent manner and was higher than that in the TSA-treated mice (Fig. 3d). The regrowth after the injection of DXR of the tumours showed more pronounced than that in TSA-treated tumours (Fig. 3e).

3.4. Effect of cell death on growth of remnant tumours

The necrosis inducer DXR was shown to enhance cell growth of remnant cancer cells *in vitro*. Thus, to examine the situation *in vivo*, we used a bilateral subcutaneous mouse tumour model. Regrowth of an injected

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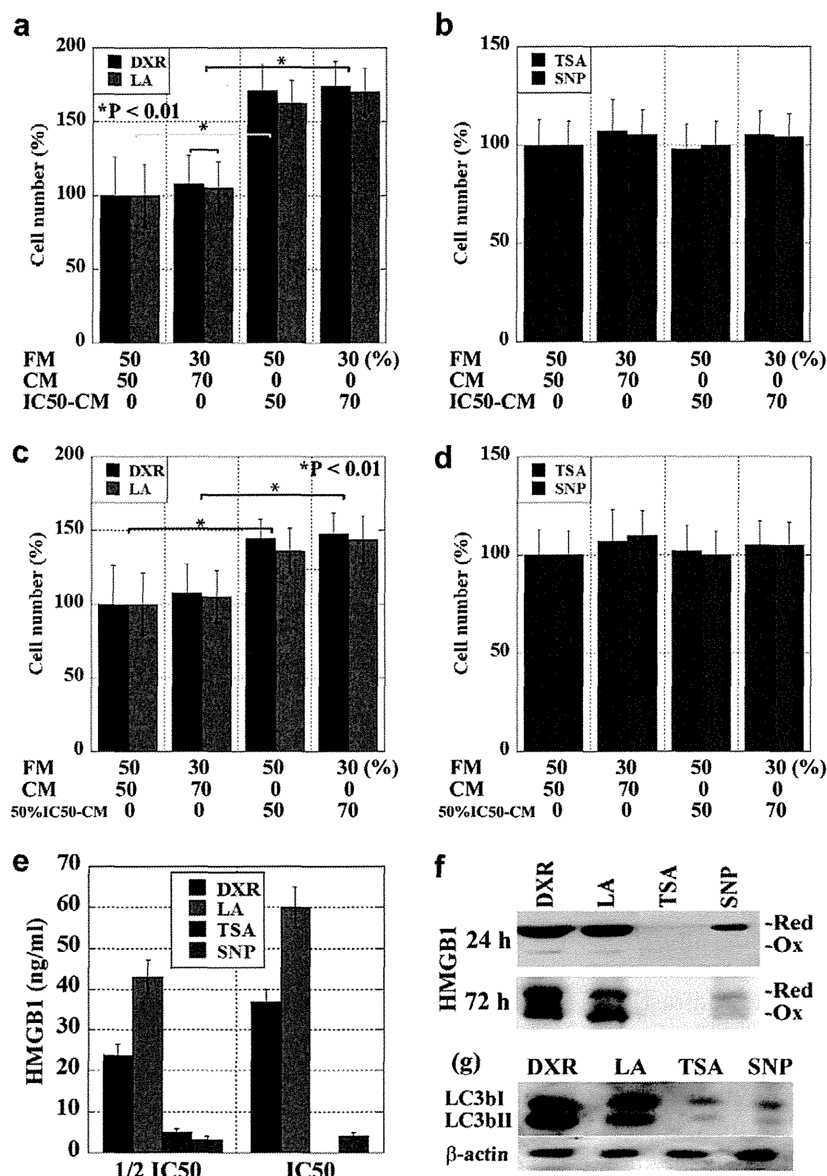

Fig. 2. Effect of culture medium (CM) from cell death inducer-treated CT26 cells on cell growth. (a–d) CT26 cells were incubated for 24 h with CM from CT26 cells treated with an 50% inhibitory concentration (IC50) of doxorubicin (DXR) or linoleic acid (LA) (a and c) and trichostatin A (TSA) or sodium nitroprusside (SNP) (b and d), mixed with fresh medium. FM, fresh medium; CM, culture medium of PBS-treated cells; IC50-CM, culture medium of cell death inducer-treated cells at the IC50 concentration. 50% IC50-CM, culture medium of cell death inducer-treated cells at 50% of the IC50 concentration. (e) HMGB1 concentration in the CM of CT26 cells treated with SNP, TSA, LA and DXR was determined by enzyme-linked immunosorbent assay (ELISA). (f) Oxidised HMGB1 was examined by sodium dodecyl sulphate–polyacrylamide gels (SDS–PAGE) in a non-reducing condition and immunoblotting. Red; reduced form, Ox, oxidised form. (g) Expression of autophagy-related microtubule-associated protein light chain (LC3) was examined by immunoblotting. Error bars represent standard deviation (SD).

tumour and the growth of contralateral tumours were monitored after injection of DXR or TSA (Fig. 4a–c). The serum concentration of HMGB1 was higher in the DXR-treated mice than in the TSA-treated mice (Fig. 4a). Regrowth of tumours injected with DXR was faster than that of the tumours injected with TSA (Fig. 4b). Moreover, growth of contralateral tumours was faster in the DXR-treated mice than in the TSA-treated mice (Fig. 4c). The difference of effects between DXR and TSA on tumour regrowth and the growth of contralateral tumours were confirmed by LL2 murine lung cancer cells in C57BL6 mice and HT29 human colon cancer cells in nude mice (Fig. 4d–f).

In the next set of experiments, anti-HMGB1 antibody was administered on days 8–10 in the same bilateral tumour model (Fig. 4g–i). The serum concentration of HMGB1 in DXR-treated mice was found to be reduced to the level observed in the TSA-treated mice (Fig. 4g). Furthermore, regrowth of tumours injected with DXR and growth of contralateral tumours in the DXR-treated mice was not statistically different from that observed in the TSA-treated mice (Fig. 4h–i).

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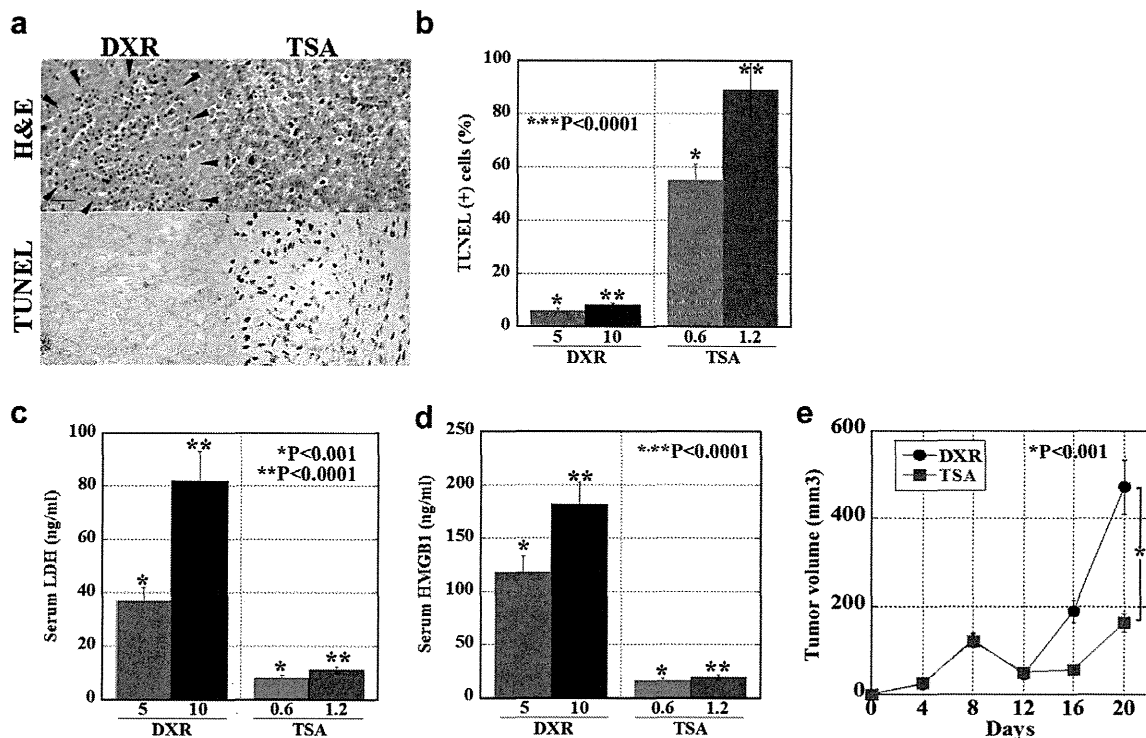

Fig. 3. Effect of cell death inducers on subcutaneous CT26 tumours. (a) Histological appearance of CT26 tumours treated by intraperitoneal (i.p.) injection of doxorubicin (DXR) or trichostatin A (TSA) at 50% inhibitory concentration (IC50) and 1/2 IC50 concentrations. H&E staining revealed necrotic changes (arrowheads). A Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labelling (TUNEL) assay showed apoptotic cells with brown nuclei. Bar represents 50 μm. (b) The frequency of apoptotic cells was examined in 2000 cells. (c and d) Serum LDH and HMGB1 concentrations (ng/ml) were determined by enzyme-linked immunosorbent assay (ELISA). (e) The growth of subcutaneous tumours treated with DXR or TSA. Error bars represent standard deviation (SD).

The effect of anti-HMGB1 antibody was examined between post- and pre-inoculation dosage (Fig. 4j–l). In comparison with the tumour-inhibitory effect of the post-inoculation treatment with the antibody, no effect was found by the pre-inoculation treatment. The effect of EP was also examined (Fig. 4j–l). EP inhibited translocation and secretion of HMGB1.[27,29] EP treatment inhibited tumour growth by both post- and pre-inoculation administration; however, the latter showed more pronounced effect. The inhibitory effect of the antibody and EP was related with HMGB1 concentrations (Fig. 4l).

3.5. Effect of cell death on metastasis

We next examined the effect of cell death on metastasis by using mouse models of metastasis (Fig. 5). In the lung metastasis model, the lung weight and number of metastatic foci were more pronounced in the DXR-treated mice than in the TSA-treated mice (Fig. 5a and b). Similarly, in the liver metastasis model, the size and number of metastatic foci were also more pronounced in the DXR-treated mice (Fig. 5c and d). However, concurrent treatment of anti-HMGB1 antibody with administration of DXR abrogated the enhancement of metastasis to both the lung and liver.

To examine the effect of cell death inducers on tumour dormancy, we used the LA-induced dormancy model (Fig. 5e).[26] Inoculated quiescent CT26 cells regrew to form tumours in the DXR-treated mice; however, no tumours were formed in the TSA-treated mice. Concurrent treatment with anti-HMGB1 antibody and DXR administration abrogated the dormancy break.

To confirm the anti-HMGB1 antibody on the signal transduction, phosphorylation of ERK1/2 was examined (Fig. 5f). Concurrent treatment of anti-HMGB1 antibody with DXR reduced the phosphorylation of ERK1/2 to the same level to those of untreated or TSA-treated tumours.

Histopathology of the metastatic foci in the lung and liver of tumours treated with DXR, TSA and DXR + anti-HMGB1 antibody was not different from that in the control. Then expressions of MIB1 and MMP2 were examined in the metastatic foci (Fig. 5g). Both expressions were higher in DXR-treated groups than those in the groups treated with TSA, anti-HMGB1 antibody or the untreated control group.

3.6. Effects of HMGB1 on innate immunity and cancer cell survival

HMGB1 plays a role in both cancer cells and immune cells via the RAGE and TLR4 receptors. Thus, we examined the effects of the balance of RAGE and TLR4 on the function of HMGB1 in cancer cells and

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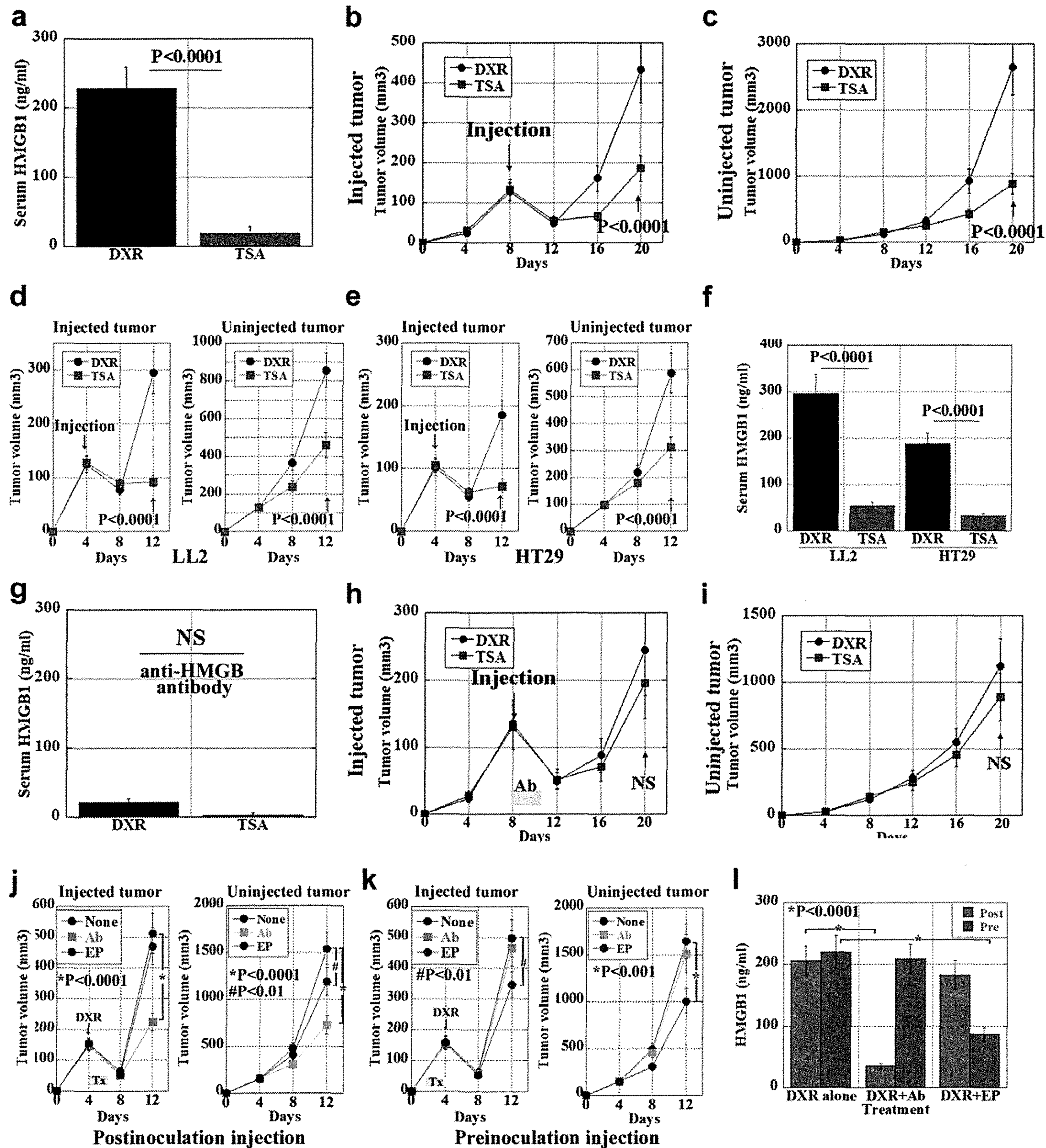

Fig. 4. Effect of cell death induction on regrowth of remnant cancer cells. (a–c) In a bilateral subcutaneous tumour model using CT26 cells in BALB/c mice (1×10^7 cells), doxorubicin (DXR) or trichostatin A (TSA) was injected into the tumour on 1 side of the mouse on day 8. (d–f) The bilateral tumour model using LL2 and HT29 cells in C57/BL6 mice and nude mice (1×10^8 cells). (g–i) In the same bilateral subcutaneous tumour model using CT26 cells in BALB/c mice (1×10^7 cells), anti-HMGB1 antibody (Ab) was administered intraperitoneally on days 8–10. (j–l) In the same bilateral subcutaneous tumour model using CT26 cells in BALB/c mice (1×10^8 cells), anti-HMGB1 antibody (Ab) or ethyl pyruvate (EP) was administered on days 4–6 (postinoculate) or on days 2–4 (preinoculate). (a, f, g, and l) Serum HMGB1 concentration was determined by enzyme-linked immunosorbent assay (ELISA) on day 9. (b, d-left, e-left, h, j-left, k-left) Regrowth of the injected tumours. (c, d-right, e-right, I, j-right, k-right) The growth of contralateral tumours. Error bars represent standard deviation (SD).

macrophages (Fig. 6). U937 monocytic cells were treated with CM from CT26 cells treated with DXR (DXR-CM) or TSA (TSA-CM) (Fig. 6a). DXR-CM treatment induced tumour necrosis factor-α (TNF-α) secretion from U937 cells, with a peak of neutralisation at 20 ng/ml of anti-HMGB1 antibody. In contrast, TSA-CM did not affect TNF-α secretion from U937 cells. U937 cells treated with HMGB1 showed induction of TNF-α secretion, with a peak at 20 μg/ml of HMGB1; however, U937 cell numbers were decreased in an HMGB1 dose-dependent manner (Fig. 6b). U937 cells expressed both RAGE and TLR4 (Fig. 6c). U937 cells with TLR4 knockdown failed to secrete TNF-α, whereas U937 cells with RAGE knockdown did not show inhibition of HMGB1-induced TNF-α secretion (Fig. 6d and e).

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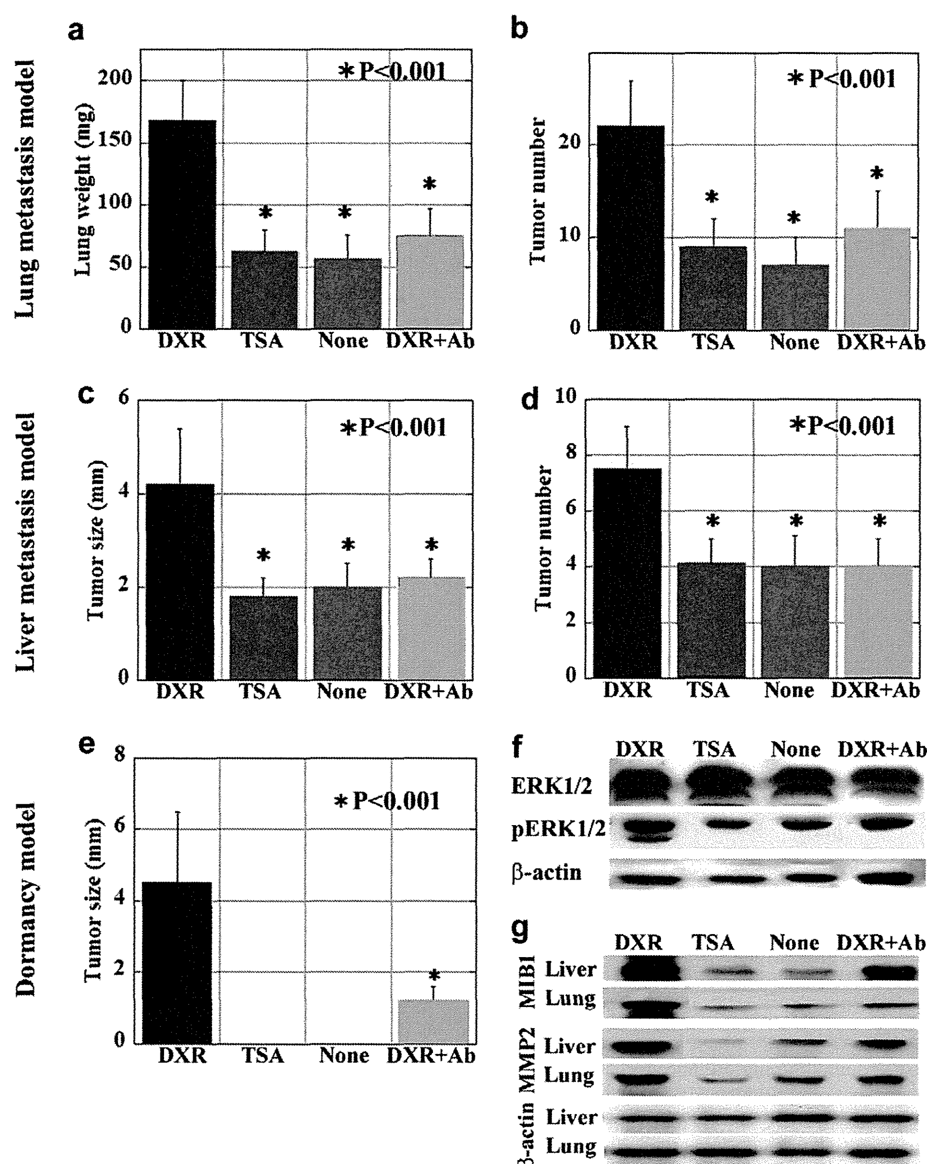

Fig. 5. Effect of cell death induction on metastasis of remnant cancer cells. CT26 cells were inoculated in the tail vein (for lung metastasis) or the spleen (liver metastasis) on day 6. doxorubicin (DXR) or trichostatin A (TSA) was injected into subcutaneous tumours on day 8. Metastasis was assessed on day 28. Anti-HMGB1 (Ab) antibody was administered on days 8 and 9. (a and b) Lung weight and number of metastatic foci of the lung. (c and d) Size and number of metastatic foci of the liver. (e) In an linoleic acid (LA)-induced dormancy model, tumour formation by LA-induced quiescent cells inoculated into subcutaneous tissue was assessed on day 28. Error bars represent standard deviation (SD). (f) Phosphorylation of extracellular signal-regulated protein kinase (ERK1/2) in the original tumour examined by immunoblotting. (g) Protein levels of MIB1 and MMP2 were examined by immunoblotting. β-Actin was examined as a loading control.

In CT26 cells, HMGB1 treatment increased IκB phosphorylation and nuclear translocation of the p65 subunit of NFκB, indicating activation of NFκB (Fig. 6f). However, RAGE knockdown inhibited HMGB1-induced NFκB activation at a more pronounced level than TLR4 knockdown did. Dual knockdown of RAGE and TLR4 resulted in total inhibition of NFκB activation by HMGB1 (Fig. 6d–g).

4. Discussion

Anti-cancer chemotherapy induces cell death in cancer cells via both necrotic and apoptotic mechanisms. Necrotic cell death permits the release of HMGB1 by loosening the nucleus.[2] Indeed, our data confirmed that necrosis inducers increase HMGB1 concentration in the CM. In contrast, the condensed nucleus found in apoptotic cells prevents the release of HMGB1. Similarly, we showed that an apoptosis inducer did not increase HMGB1 concentration in the CM. This difference in HMGB1 concentration was also observed in a mouse model; treatment of CT26 tumours with the necrosis inducer, but not the apoptosis inducer, increased serum HMGB1 levels.

HMGB1 is known to act as a growth factor for cancer cells,[9,16,17] because HMGB1 activates mitogen-acti-

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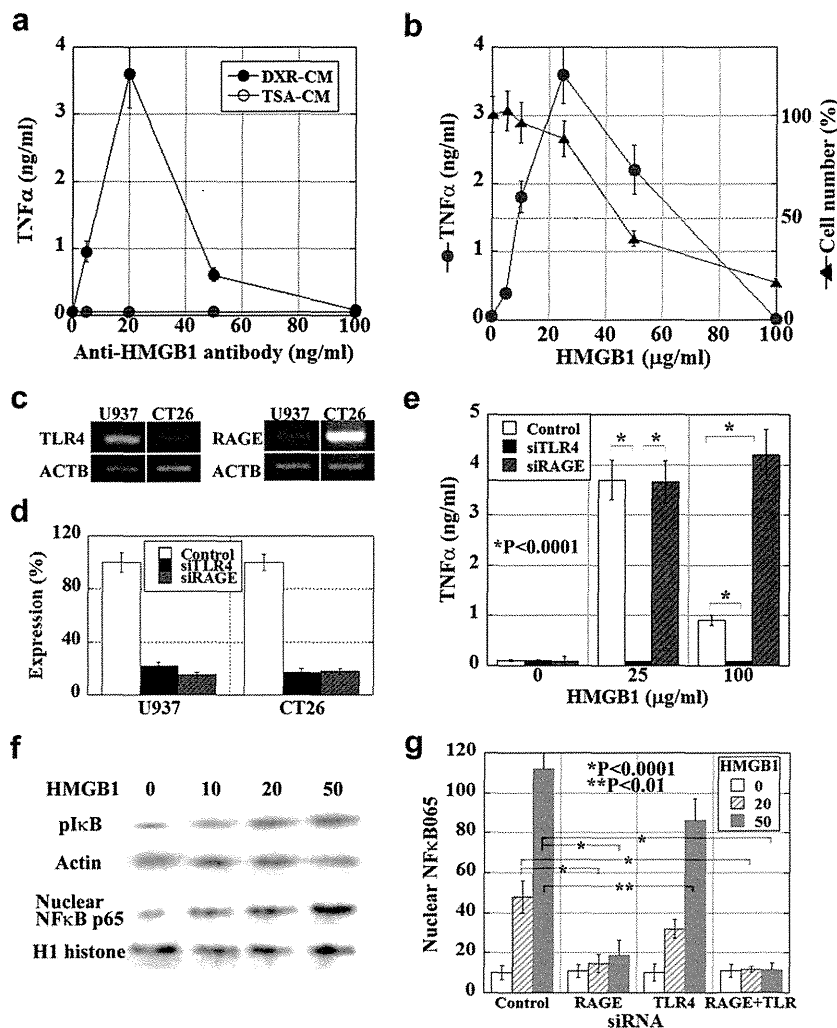

Fig. 6. Effect of HMGB1 on innate immunity and cancer survival. (a) U937 human monocytic leukaemia cells were incubated with CT26 cell culture medium (CM) treated with doxorubicin (DXR-CM) or trichostatin A (TSA) (TSA-CM), and HMGB1 was neutralised by an anti-HMGB1 antibody. TNF-α concentration in the CM was determined by enzyme-linked immunosorbent assay (ELISA). (b) U937 cells were treated with HMGB1, and cell number and CM TNF-α concentration were examined. (c) Expression of RAGE and TLR4 in U937 and CT26 cells was determined by reverse transcription-polymerase chain reaction (RT-PCR). (d) Effect of Small interfering RNA (siRNA) directed against RAGE and TLR4 on the expression of RAGE and TLR4 in U937 and CT26 cells. (e) Effect of knockdown of RAGE or TLR4 on TNF-α secretion by U937 cells. (f) Levels of phosphorylated IκB and nuclear NFκB p65 were examined by immunoblotting. Actin and H1 histone levels served as internal controls. (g) Effect of siRNA directed against RAGE and TLR4 on the levels of nuclear NFκB p65 in CT26 cells. Error bars represent standard deviation (SD).

vated protein kinase, and thereby enhances proliferation and invasion via activation of the RAGE receptor.[9,30] HMGB1 also activates mammalian target of rapamycin (mTOR) to enhance cancer cell survival.[31] HMGB1 acts as an inflammatory cytokine and as a suppressor of monocyte lineages, including lymph sinus macrophages, liver Kupffer cells and dendritic cells.[23,32–34] Furthermore, HMGB1 induces apoptosis in monocyte lineages, and this causes a reduction of anti-cancer immunity and an increase in the metastatic capacity of cancer cells.

Paradoxically, our data showed increased growth of remnant cancer cells and metastasis to the liver and lung in the necrosis inducer-treated mice. In contrast, this increase of tumour regrowth and metastasis was not found in the mice treated with an apoptosis inducer. Moreover, treatment with anti-HMGB1 antibody abrogated the increase of tumour regrowth and metastasis.

We previously reported that LA induces quiescence of cancer cells by the long-term treatment.[13] Furthermore, quiescent cancer cells adopt a dormant status in inoculated mice. Our data showed that induction of necrosis in a visible tumour activated LA-induced dormant cancer cells inoculated in the contralateral side of the back. In contrast, treatment with an apoptosis inducer did not activate the dormant cells. The mechanisms responsible for induction and breakage of the cancer dormancy are still unclear. However, our results suggest that HMGB1 released from necrotic cells by chemotherapy results in a dormancy break of hidden cancer cells.

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Recently, HMGB1 has been shown to present DNA to TLRs to activate innate immunity.[6,7] This suggests that HMGB1 is capable of enhancing anti-cancer immunity; however, high levels of HMGB1 suppress monocyte lineages, thereby inhibiting anti-cancer immunity.

From our findings, we consider that apoptosis inducers are important for anti-cancer chemotherapy. Chemotherapy using necrosis inducers can lead to obvious reduction of tumours by necrosis; however, it may enhance regrowth and metastasis of cancer cells that have survived treatment, and activate scattered cancer cells that are in a dormant status. Alternatively, HMGB1 released from necrotic cancer cells may activate the innate immune system to promote host anti-cancer immunity. Thus, the role of chemotherapy-associated HMGB1 is thought to depend on the balance of these opposing effects of HMGB1. To confirm it, we examined the effect of HMGB1 on TNF-α secretion in U937 macrophages and NFκB activation in CT26 colon cancer cells. Our results suggest that RAGE-related HMGB1 effects are cancer activated and immunosuppressive. RAGE activation in CT26 cells induced NFκB activation, which enhances cancer cell survival. In contrast, RAGE activation in U937 cells reduced cell numbers and inhibited TNF-α secretion. RAGE activation induces apoptosis of U937 cells via c-Jun N-terminal kinase phosphorylation.[23] In contrast to RAGE, TLR4-related HMGB1 activated the immune system. TLR4 activation by HMGB1 induced TNF-α secretion in U937 cells, whereas TLR4 showed a restricted effect on NFκB activation in CT26 cells. These findings suggest that RAGE suppression is effective in avoiding regrowth and metastasis of cancer cells by necrosis-related HMGB1 in chemotherapy.

Our results suggest that to avoid cancer reactivation by HMGB1 released from necrotic cancer cells, it may be important to use apoptosis inducers as an alternative to necrosis inducers, or target RAGE by using necrosis inducers. Recently, a number of RAGE-targeting methods have been proposed.[1] Such proposals may be considered for future chemotherapy protocols, to establish more effective immunogenic chemotherapy.[7]

Conflict of interest statement

We declare that there is not any Financial Support or Relationships which may pose a conflict of interest in the contents of the submitted manuscript. All authors have approved the comments.

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Trks are novel oncogenes involved in the induction of neovascularization, tumor progression, and nodal metastasis in oral squamous cell carcinoma

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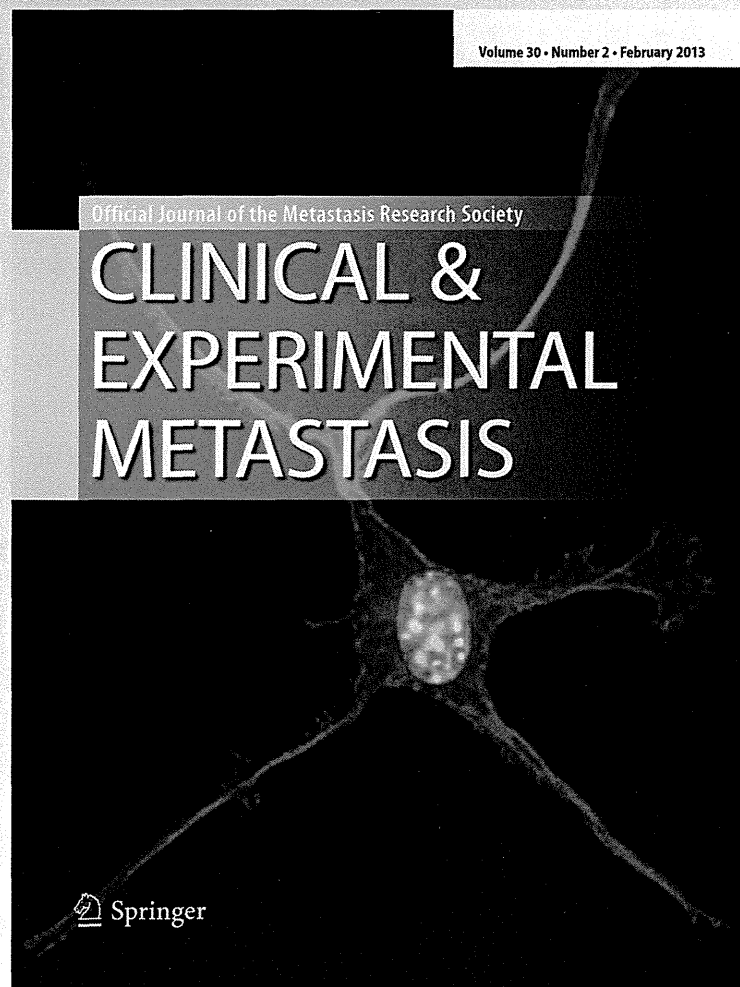

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