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Research Paper

Combination Therapy of Heparin–Deoxycholic Acid Conjugate and Doxorubicin against Squamous Cell Carcinoma and B16F10 Melanoma

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Purpose. Our previous study confirmed that heparin–deoxycholic acid conjugate (HD) had a potent antiangiogenic effect and safety to use for long-term treatment. Herein, the combined therapeutic effect of HD and doxorubicin (DOX) was evaluated against squamous cell carcinoma (SCC7) and B16F10 melanoma.
Methods. The inhibitory effect of cell proliferation and cellular uptake of HD was studied in SCC7 and B16F10. The combination effects of HD and DOX were evaluated by measuring cytotoxicity and apoptosis as well as tumor growth and apoptosis *in vivo* against SCC7 and B16F10 tumor-bearing mice.
Results. HD displayed potent inhibitory effect on SCC7 and B16F10 cell proliferation, but it showed a low cytotoxic effect. Concurrent treatment of HD and DOX displayed enhanced cytotoxic effects and apoptosis on SCC7 and B16F10. The cellular uptake of HD and DOX was affected by the collective cytotoxic effects of these two drugs: each drug suppressed the tumor growth, and their combined treatment enhanced apoptosis and collectively inhibited the tumor growth of SCC7 and B16F10 *in vivo*.
Conclusion. These results demonstrated that HD with cytostatic and antiangiogenetic activities, enhanced the antitumor activity of DOX against SCC7 and B16F10, and the combined treatment of these two drugs might have enhanced therapeutic efficacy.

KEY WORDS: apoptosis; B16F10; doxorubicin; heparin–deoxycholic acid conjugate; SCC.

INTRODUCTION

Heparin is a highly sulfated natural polysaccharide, which is mainly comprised of alternating units of sulfated glucuronic acid and glucosamine derivatives (1). Heparin, a widely used anticoagulant, is administered to prevent deep vein thrombosis (DVT) and pulmonary embolism (PE) in high-risk patients (2,3). It has been reported that heparin inhibits tumor growth and angiogenesis, because it can modulate the binding of angiogenic growth factors (i.e. VEGF and bFGF) and cytokines, and regulates various enzymatic activities (4–6). In addition, it has been known that heparin attenuates metastasis by blocking selectin-mediated intercellular interactions, for instance, tumor-platelet and tumor-endothelial cells (7–10). Recently, heparin was demonstrated to inhibit the proliferation of smooth muscle cells and hepatoma cells (11–14). It can also induce apoptosis in human peripheral neutrophils (15) and lymphoblasts (16) and nasopharyngeal carcinoma CNE2 cells (17).

Heparin has a variety of excellent biological effects; however, the use of heparin is strictly limited at high dosages and for extended periods of time because of its strong anticoagulant activity. Heparin-induced side effects in clinics include hemorrhages, HIT (heparin-induced thrombocytopenia), and osteoporosis (18,19). To overcome the side effects induced by high dose or long-term heparin treatment, carboxyl-reduced heparin, *N*-*O*-desulphated/*N*-resulphated heparin, *N*-desulphated/*N*-reacetylated heparin (20), heparin–steroid conjugate (21), heparin-carrying polystyrene (22), and neoheparin (23) have been developed. These chemically modified heparin derivatives reduced the risk of hemorrhage and prevented metastasis, angiogenesis, tumor growth or cancer cell proliferations. However, high dosages (above 40 mg kg^{-1} day^{-1}) are required to achieve an antitumor effect.

In the previous studies, we prepared heparin–deoxycholic acid conjugate (HD) that was found to be safe with respect to long-term treatment in a mouse model (24,25). This heparin

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ABBREVIATIONS: BFGF, basic fibroblast growth factor; CLSM, confocal laser scanning microscopy; DOX, doxorubicin; DVT, deep vein thrombosis; FITC, fluorescein isothiocyanate; HUVECs, human umbilical vein endothelial cells; PE, pulmonary embolism; SCC7, squamous cell carcinoma; VEGF, vascular endothelial growth factor.

derivative effectively prevented the cell proliferation of SCC7 and human umbilical endothelial cells (HUVECs) (25), and it suppressed angiogenesis, thereby inhibiting the tumor growth in the SCC7 mouse model (26). Therefore, HD with antiproliferative and antiangiogenic effects against tumor and endothelial cells might be more suitable for cancer treatment, compared to the previously reported heparin derivatives.

However, cytostatic and antiangiogenic drugs should be required for long-term treatment compared to cytotoxic drugs. It is also possible that their use for long periods can cause problems because genetically abnormal tumor cells or tumor endothelial cells become drug resistant (27). Also, recently, many clinical studies have been performed to evaluate the combination effect of cytotoxic drugs and molecular targeted cytostatic or antiangiogenic drugs. In this study, we evaluated the combination effects of HD and DOX on cytotoxicity and induction of apoptosis as well as the regression of subcutaneously implanted SCC7 and B16F10 tumors. Also, we evaluated the inhibition effect of HD and DOX on the tumor growth of SCC7 and B16F10 in the mouse model.

MATERIALS AND METHODS

Chemicals

Unfractionated heparin (UFH, 167 IU/mg) of average molecular weight, ca 12,000 Dalton, was purchased from Pharmacia Hepar Co. (Franklin, OH). HD was prepared by reacting heparin with the bile acid analogue, *N*-deoxycholylethylamine, as previously described (25,26). Dimethyl sulfoxide (DMSO), doxorubicin (DOX), fluorescein isothiocyanate (FITC), Triton X-100, trypsin-EDTA solution, and propidium iodide were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents were of analytical grades and used without further purification.

Cell Proliferation and Cytotoxicity Assays

Squamous cell carcinoma (SCC7) and B16F10 melanoma were obtained from American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% FBS and 1% penicillin–streptomycin at 37°C in a humidified 5% CO_2–95% air atmosphere.

In the proliferation assay, cells were plated into 12-well culture plates at a density of 2×10^4 cells/well, and allowed to attach overnight. On the following day, cells (confluency of 60 to 70%) were treated with HD at different concentrations (5, 10, 50, 100 μg/ml). After 24 h, cells were harvested, stained with trypan blue and the cell number was counted using a hematocytometer. Growth inhibition was calculated as follows:

$$\%\,\text{inhibition} = \left[1 - \left(\frac{\text{net cell number in presence of HD}}{\text{net cell number in control}}\right)\right]$$

The cytotoxic effects of HD in the presence of DOX were assayed by MTT colorimetric assay. Cells were seeded at a density of 5×10^3 cells/well in 96-well flat-bottomed plates for 24 h. Cells (confluency of nearly 80%) were washed twice with PBS and incubated with various concentrations of HD (5, 10, 50, 100 μg/ml) in the presence of DOX (0.5 μg/ml) for 24 h at 37°C. The concentration of DOX at 0.5 μg/ml was chosen because this concentration had much lower IC_{50} values of DOX [IC_{50} values of DOX against SCC7 and B16F10 cells were 2.6 μM (28) and approximately 0.41∼1.03 μM (29,30), respectively] and also showed minor or moderate cytotoxic effects against SCC7 and B16F10 cells. The cells were washed twice with PBS to eliminate the remaining drugs and the fresh culture medium was added. MTT solution (20 μl, 5 mg/ml in PBS) was then added to each well and the cells were incubated further for 4 h at 37°C. The media were removed and the cells were dissolved in DMSO. Ultraviolet absorbance at 570 nm was measured with a microplate reader (VERSAmax™, Molecular Devices Corp., Sunnyvale, CA). The data were expressed as the percentage of viable cells compared to the control group.

Cellular Uptake of FITC-Labeled Heparin–DOCA

Cells were seeded at a density of 1×10^4 cells/well in an eight-well chamber slide and kept overnight. The cells were treated with FITC-labeled heparin (500 μg/ml), FITC- HD (500 μg/ml) and DOX (0.5 μg/ml), respectively. After incubation in 10% serum medium for 12 h, respectively, the cells were washed with cold PBS three times and fixed in 1% paraformaldehyde solution for 1 h at 4°C. The slides were covered with a mounting medium (SuperMountR, InnoGenex) and images were taken with a confocal laser scanning microscopy (CLSM 510, CARL-ZEISS). The fluorescence images were observed in two channels: green (expressing FITC-HD; excitation 490 nm, emission 520 nm) and red (expressing DOX; excitation 570 nm, emission 590 nm).

Apoptosis Assay

Cells were seeded at a density of 2×10^4 cells/well in 24-well plates and allowed to attach on plates. HD (100 μg/ml) was treated to cells with or without DOX (0.5 μg/ml). After 24 h, cells were washed with fresh culture medium four times to remove treated drugs and fixed in 1% paraformaldehyde. To detect apoptosis by changes in the nuclear morphology of cells, cells were stained with the fluorescent DNA dye 4′,6-diamidine-2′-phenylindol dihydrochloride (DAPI) (Calbiochem, San Diego, CA) solution for 10 min. Following washing with PBS, the nuclear morphological changes of cells were evaluated by fluorescence microscope.

Flow cytometry was used to quantify apoptotic cells as previously reported (31). Cells (3×10^5 cells/well) were treated with HD (100 μg/ml) with or without DOX (0.5 μg/ml) for 24 h, and then trypsinized, washed with PBS twice and kept in 70% ethanol overnight −4°C. Propidium iodide/RNase was added, cells were incubated for 30 min at room temperature and then were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, NJ). The data were plotted on FL2-H histograms and the sub-diploid cells were counted and designated as apoptosis.

Combination Therapy of HD and Doxorubicin against SCC7 and B16F10

Male C3H/HeN and C57BL/6 mice (7 weeks old, Charles River Laboratories Inc.) were given dorsal subcuta-

neous injections of 1×10^6 cells/mouse. Care and maintenance of animals were done in adherence to Institutional guidelines of the Institutional Animal Care and Use Committee (IACUC). When tumors reached 50 to 100 mm^3, the mice were given intravenous injections of 100 μl of saline containing HD (5 mg/kg/3 day) and DOX (5 mg/kg/6 day). Tumors were measured daily with calipers. On the tenth day, mice were sacrificed and tumors were removed. Treatment groups contained six to seven mice. Tumor tissues were isolated from three representative treated and untreated tumor-bearing mice Tumor tissues were isolated from three representative treated and untreated tumor-bearing mice. To evaluate apoptosis *in vivo*, terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay was performed on paraffin-embedded sections by using the ApopTag®peroxidase *in situ* apoptosis detection kit (Chemicon, CA).

Statistics

The statistical significance of differences between experimental and control groups was determined using one-way ANOVA. P values < 0.05 were considered significant and are indicated in figures by asterisks.

RESULTS

Synthesis and Characterization of HD

The synthesis and characterization of HD were carried out as previously reported (24,25). Briefly, a primary amine group was introduced to DOCA using ethylenediamine and then followed by chemical coupling reaction with heparin. The produced HD confirmed that the amide linkages between heparin and DOCA were formed at the wavelength of 1,665 cm^{-1} in FT-IR spectra. On the other hand, though it had no absorption in FT-IR spectra for heparin, the peak near the wavelength of 2,360 cm^{-1} represents the stretching vibration of intrinsic sulfonamide ($-NHSO_3$) in heparin. The presence of DOCA in heparin was confirmed by the characteristic peaks of bile acid appearing at 0.65~2.3 ppm. The new amide linkages between heparin and DOCA and the intrinsic sulfonamide in heparin appeared at 8.04 and 5.3 ppm in the ^{1}H-NMR spectra, respectively. The degree of substitution (DS) of DOCA to heparin controlled by the feed mole ratio of DOCA–NH_2 was calculated by measuring the remaining carboxylic groups in HD conjugates using the titration method. The average molecular weight of HD conjugates was 16,990, based on the calculation from the degree of substitution (DS, 11.1) that indicates the number of deoxycholic acids per heparin (25). Throughout the modification of heparin's carboxyl groups with DOCA–NH_2, the anticoagulant activity of HD conjugates was decreased up to 23% compared to that of the native heparin because the carboxyl groups of heparin play an important role in anticoagulant activity. After chemical modification of heparin with DOCA–NH_2, the negative charge of HD conjugates was −55.9 mV, and much lower than that of heparin (−75~−100 mV).

Antiproliferative Effect and Cytotoxicity of HD

To determine the antiproliferative effect of HD, we used various concentrations of HD for 48 h, and counted the number of growing cells. The antiproliferative effect of HD against SCC7 and B16F10 was assessed by the decreased number of growing cells compared to the number of growing cells in the control (no-treatment) group. We found that the inhibitive effect on the cell proliferation of SCC7 and B16F10 was increased by increasing the concentration of HD. We found that HD exhibited slightly greater antiproliferative effect on B16F10 than on SCC7, although there were no statistical differences between two cell lines. As shown in Fig. 1a, HD at 100 μg/ml inhibited cell proliferation of SCC7 and B16F10 by 27.6 and 30.6%, respectively.

The cytotoxic effect of HD against SCC7 and B16F10 cells was evaluated by a MTT colorimetric assay. HD at 100 μg/ml slightly decreased cell viability in the two cell lines, compared

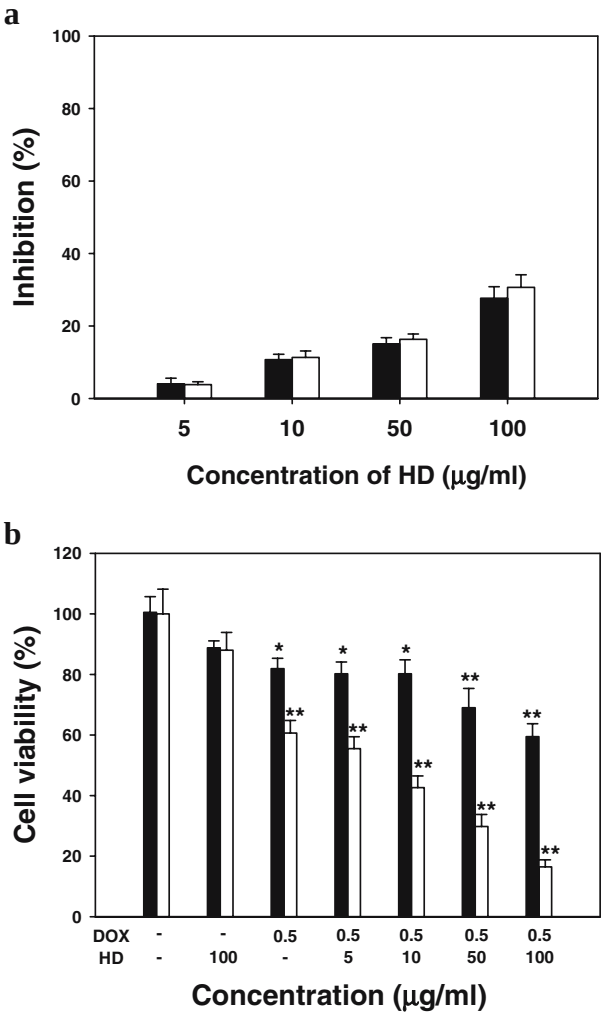

Fig. 1. Cell proliferation and cytotoxic effects. **a** Antiproliferative effect of HD on squamous cell carcinoma (SCC7, ■) and B16F10 melanoma cells (□) at 24 h. Data are presented as means±SE ($n = 3$). **b** Cytotoxic effect of HD at different concentrations with doxorubicin (0.5 μg/ml) on SCC7 (■) and B16F10 (□). Data are presented as means±SD ($n = 6$~8). (**$P < 0.01$, and *$P < 0.05$ versus control).

to the control (Fig. 1b), indicating that HD was not cytotoxic against SCC7 and B16F10 cells. DOX decreased cell viabilities by 18.0 and 39.4% in SCC7 and B16F10, respectively, compared to the control group. When HD (at 100 μg/ml) and DOX (at 0.5 μg/ml) were concurrently treated, the cell viabilities were significantly decreased by 40.5 and 83.5% in SCC7 and B16F10, respectively. From the MTT results, the IC_{50} of the combined modality against SCC7 was above 0.5 μg/ml of DOX and 100 μg/ml of HD, whereas IC_{50} of the combined modality against B16F10 melanoma was nearly 0.5 μg/ml of DOX and 7.3 μg/ml of HD. From these results, HD conjugate reduced cell growth by proliferative inhibition, rather than by the induction of cell death, and therefore, the combined treatment using these two drugs was significantly effective in increasing cytotoxicity against SCC7 and B16F10.

Cellular Uptake of FITC-Labeled HD *In Vitro*

Cellular uptake of DOX, FITC-heparin and FITC-HD in SCC7 and B16F10 were evaluated by using confocal laser scanning microscopy (CLSM). As shown in Fig. 2, most of DOX was localized in nuclei of two tumor cells. Cellular uptake of DOX in B16F10 was much higher than that in SCC7 at 12 h, and thus it was likely that DOX was more cytotoxic and sensitive against B16F10 than against SCC7 due to higher cellular uptake. FITC-heparin was hardly taken up, whereas FITC-HD was highly taken up in both SCC7 and B16F10 cells at 12 h. Unlike DOX, FITC-HD was mostly distributed into cytoplasm in both SCC7 and B16F10 cells. In addition, the cellular uptake of FITC-HD in both SCC7 and B16F10 was almost similar.

Apoptosis and Flow Cytometric Analysis

The effect of HD on apoptosis was evident by DAPI staining of cell nuclei. Cell shrinkage, condensed or fragmented nuclei represent cells undergoing apoptosis. In Fig. 3, in the control and in HD treated cells, condensed and fragmented nuclei of cells were not observed, indicating that HD alone dose not induce apoptosis. However, condensed and fragmented nuclei were observed in SCC7 and B16F10 at 24 h after the treatment of DOX. The combined treatment of HD and DOX showed potent induction of apoptosis. In addition, more apoptotic cells were observed in B16F10 cells treated with HD and DOX rather than in SCC7. Apoptosis induction by HD with or without DOX was determined by flow cytometry. As shown in Fig. 4a and b, HD did not induce apoptosis against two cell lines (less than 5%). Inductions of apoptosis by DOX were 14.4 ± 3.4% on SCC7 and 30.3 ± 4.6% on B16F10. The combined treatment of DOX and HD significantly augmented apoptosis induction in both SCC7 (24.5%) and B16F10 (64.0%), and apoptosis induction was increased by 1.7-fold in SCC7 cells and 2.1-fold in B16F10 cells when compared to the cells treated by DOX. The combination therapy using HD and DOX clearly showed enhanced cytotoxicity and apoptosis induction against SCC7 and B16F10 cells compared to when either one of HD or DOX treatments was used alone.

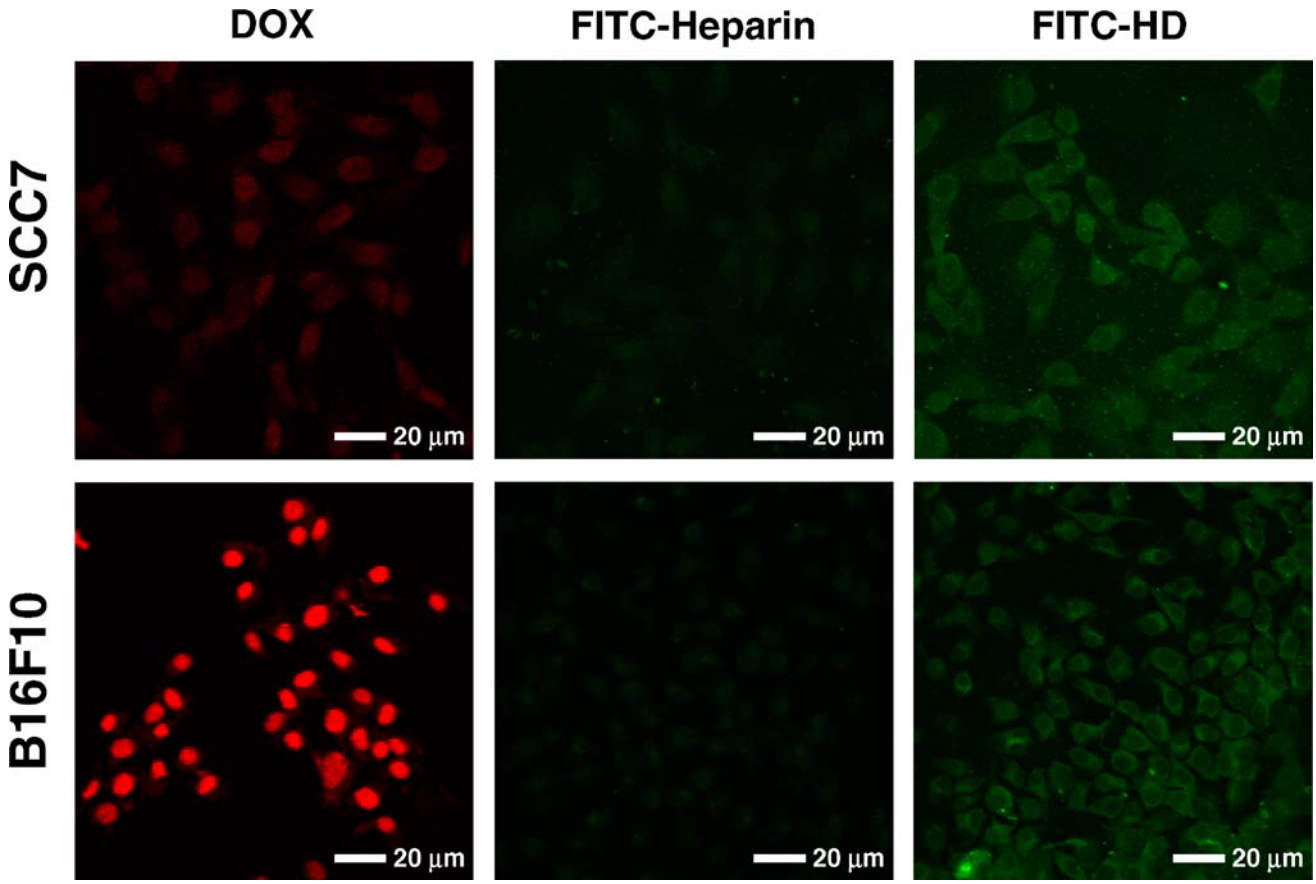

Fig. 2. Cellular uptake of DOX, FITC-heparin and FITC-HD on SCC7 and B16F10 by confocal laser scanning microscopy (CLSM).

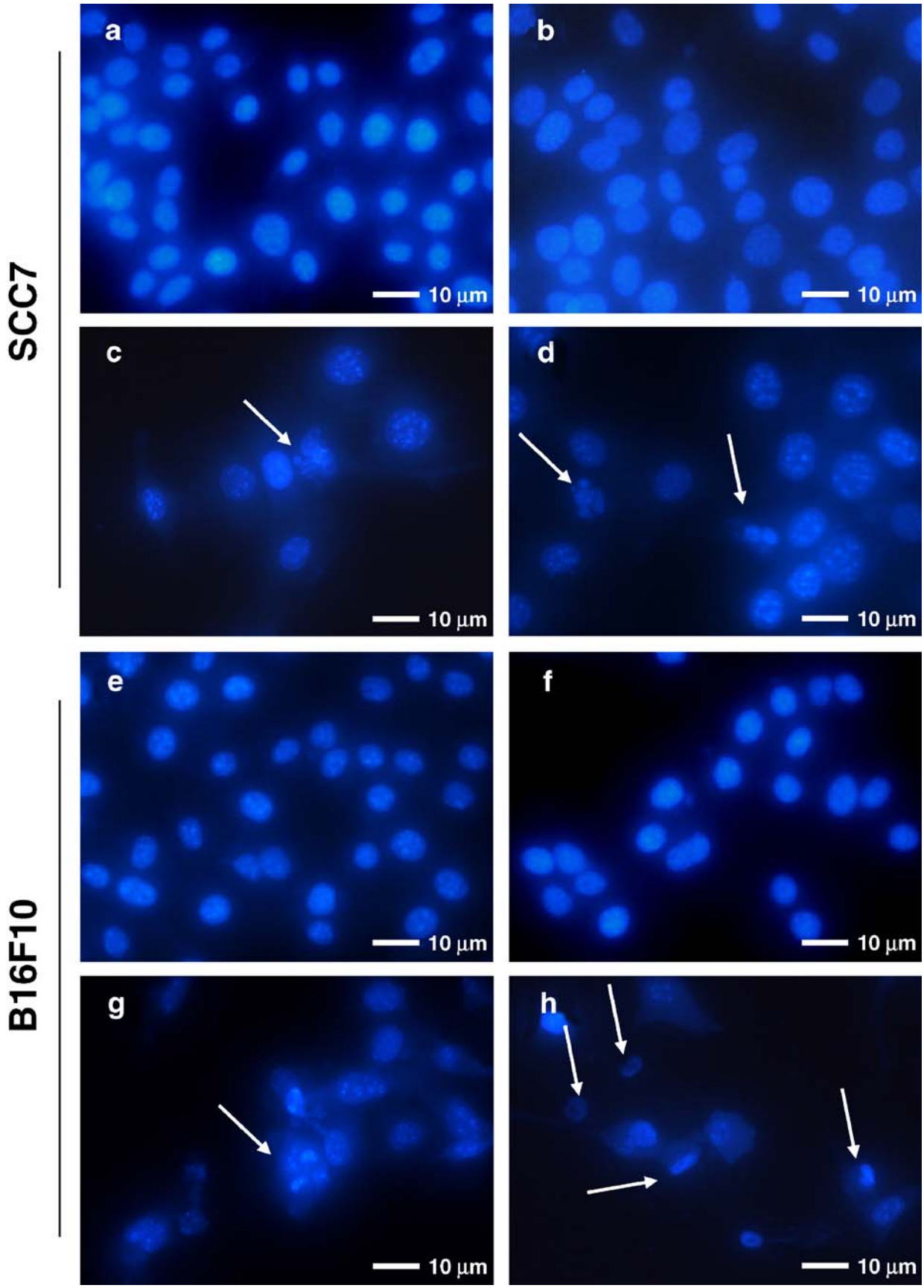

Fig. 3. Detection of apoptosis by DAPI stain in (**a**–**d**) SCC7 and (**e**–**h**) B16F10 melanoma. **a** and **e** Control; **b** and **f** HD (100 μg/ml); **c** and **g** DOX (0.5 μg/ml) and **d** and **h** HD (100 μg/ml) + DOX (0.5 μg/ml) at 24 h. *Arrows* represent the condensed or fragmented nuclei of cells.

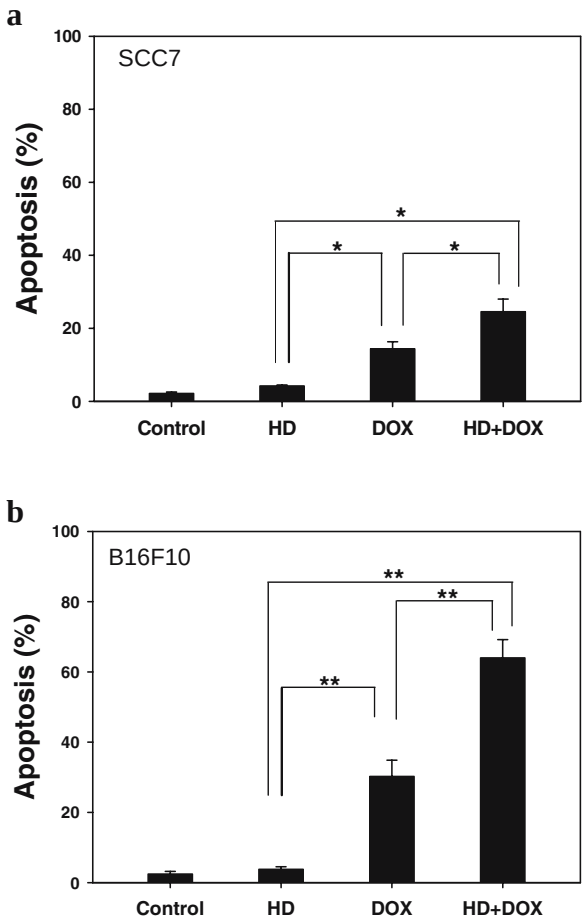

Fig. 4. Induction of apoptosis by HD (100 μg/ml) and/or DOX (0.5 μg/ml) in **a** SCC7 and **b** B16F10 cells. Cells were harvested after 24 h incubation for quantification of apoptosis by flow cytometry. Apoptosis was quantified as percent of subdiploid cells. Data are presented as means±SD ($n=3$). (**$P<0.001$ versus control; *$P<0.05$).

Antitumor Effects of HD and Doxorubicin on SCC7 and B16F10 Tumor Models

Both treatments using either HD (5 mg/kg/3 day) or DOX (5 mg/kg/6 day) alone inhibited the tumor growth in SCC and B16F10 implanted mice. However, HD alone suppressed tumor volumes by 54.0% on SCC7 and by 51.6% on B16F10 cells (at day 10), whereas DOX alone inhibited tumor volumes by 72.4% on SCC7 and by 80.8% on B16F10 ($P<0.001$ vs control) at day 10. The combined treatment was more effective than the respective single treatments [tumor volume of the control at day 10, 13.0% on SCC7 ($p<0.001$ vs control) and 5.1% on B16F10 ($p<0.001$ vs control)] (Fig. 5a and b. Comparison among treated groups showed that the combined treatment of HD and DOX against SCC7 and B16F10 tumors showed statistically improved antitumor effect, respectively.

Apoptotic cells were rarely detectable in the control and HD treated groups, whereas apoptotic tumor cell nuclei (brown color) were clearly detected in DOX-treated SCC7 and B16F10 tumor tissues, as shown in Fig. 6. Furthermore, more apoptotic cells were found in tumor tissues treated concurrently with HD and DOX. These results demonstrated that the concurrent treatment of HD with DOX provides potent anti-tumor effects in SCC7 and in the B16F10 implanted murine model.

DISCUSSION

In the previous studies, we have developed HD with low anti-coagulant activity, and demonstrated that it had antiproliferative effect against endothelial cells and that it could be used as an angiogenesis inhibitor for cancer therapy because HD could inhibit bFGF-induced angiogenesis by interfering with the biological activities of bFGF. These pleiotropic activities of HD resulted in the significant antitumor effects. When HD was used with DOX in this study, it enhanced the antitumoral effect of DOX by enhancing cytotoxicity and apoptosis induction in SCC7 and B16F10 *in vivo* models.

We had already demonstrated that HD showed an inhibition effect on endothelial cell proliferation, and that it was more effective than heparin. In the present study, we demonstrated the antiproliferative effect of HD against

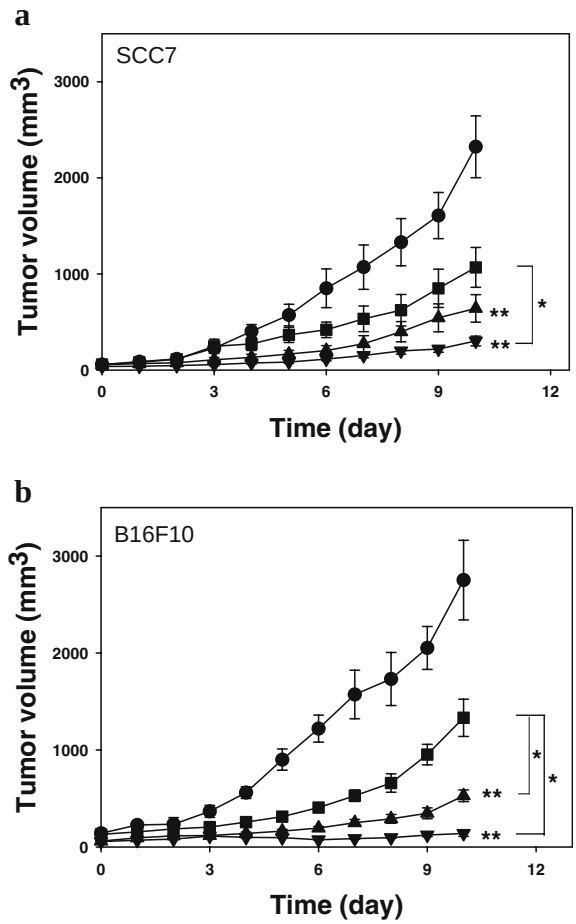

Fig. 5. Antitumor effects of HD with or without DOX against **a** SCC7 and **b** B16F10 melanoma. Mice were given s.c. injection with SCC7 or B16F10, and when tumor volumes were 50 to 100 mm^3, the mice were given i.v. injection with (●) normal saline, (■) HD (5 mg/kg/3 day), (▲) DOX (5 mg/kg/6 day), and (▼) HD (5 mg/kg/3 day) plus doxorubicin (5 mg/kg/6 day). Each group contained six to seven mice. The tumor sizes were measured daily. Data are presented as means±SE. (**$P<0.001$ versus control; *$P<0.05$).

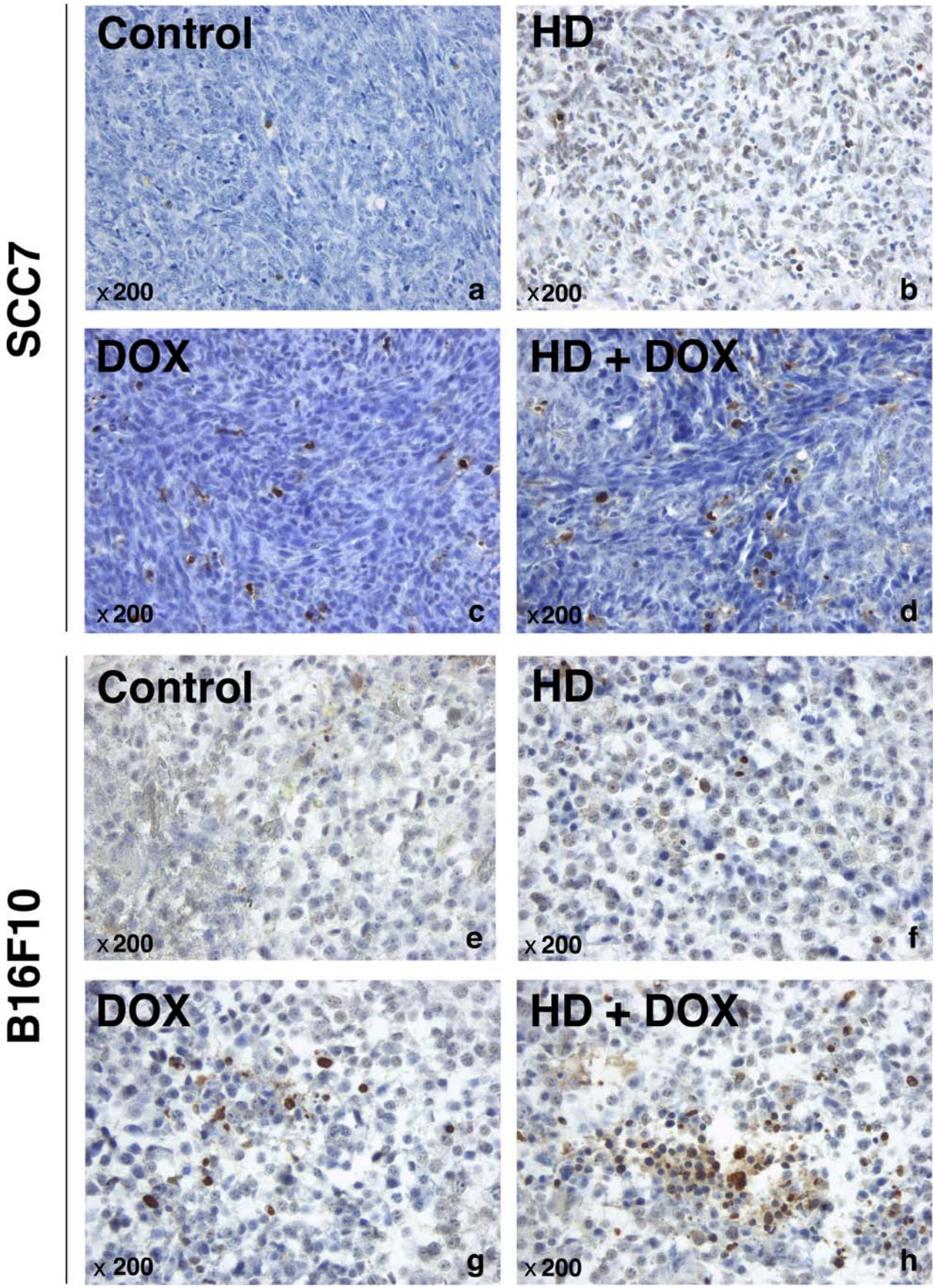

Fig. 6. *In vivo* TUNEL assay against s.c. induced (**a**–**d**) SCC7 and (**e**–**h**) B16F10 tumors. *Brown color* indicates apoptotic cells in tumors.

SCC7 and B16F10 by showing that the number of growing tumor cells was significantly decreased when the concentration of HD is increased. As confirmed in our previous study, HD exhibited more potent antiproliferative effect against endothelial cells and SCC7 cells. Compared to heparin, it expected that more potent antiproliferative effect of HD is correlated with its internalization into tumor cells. Indeed, we observed that FITC-labeled HD was highly internalized, and mostly distributed into the cytoplasms of two tumor cells, whereas FITC-heparin was hardly taken-up by cells. Their different physicochemical properties offer different cellular uptake capacity, thus affecting cell proliferation. There are several articles to show that the internalized heparin could affect cellular processes such as cell proliferation inhibition. The internalized heparin inhibits cell proliferation by inhibiting other important transcription factors including c-myb, Oct-1, c-Jun/c-Fos/AP-1 transactivation, which presumably play a role in cell proliferation (32–34). Based on these facts, we conclude that the internalized heparin–DOCA alone may interact with nuclear proteins, and alter the activities of transcription factors, thereby suppressing cell proliferation.

Due to the highly negative charge powered by many sulfates and carboxyl groups, heparin is scarcely taken up by cells through cell membranes. As previously reported, more anionic biopolymers do not enable internalization through the negatively charged cell membrane (35). However, the HD conjugate has much lower negative charge than heparin, and it showed amphiphilicity due to the presence of a hydrophobic moiety such as DOCA analogue. The increased amphiphilicity of HD conjugate might have improved the permeability via passive diffusion across the cell membranes (36). In addition, HD conjugate formed self-assembled nanoparticles, which may alter non-specific interactions with the cell surface making it more likely to be taken up by endocytotic process compared to heparin. As a result, the amphiphilic features of HD seem to be important for cellular internalization, and thus for determining cell proliferation inhibition.

We performed the combination therapy using HD as a cytostatic agent and DOX as a cytotoxic drug against SCC7 and B16F10 tumors. HD as a cytostatic drug revealed its potent inhibition effect on the proliferation of tumor cells and tumor growth *in vivo*, but did not appear cytotoxic nor did it induce apoptosis *in vitro* and *in vivo*, indicating that long-term treatment is periodically required to obtain antitumor effects by HD. DOX, on the other hand, being a cytotoxic drug, exerted cytotoxic effects, and induced apoptosis *in vitro* and *in vivo*, and significant tumor growth inhibition. When they were combined together, the significantly enhanced therapeutic effects included increased cytotoxicity, apoptosis induction, thus resulting in tumor suppression against SCC7 and B16F10 tumors. These results suggest that the combination therapy of HD and DOX significantly enhanced their respective therapeutic efficacies.

In the combination therapy of HD and DOX, herein, therapeutic effects are more effective against B16F10 cells than against SCC7 cells. This difference in therapeutic effects might be mainly due to the difference in the cellular uptake of DOX into tumor cells. As seen in the above result, HD exhibited similar cellular uptake, and thus exerted antiproliferative and antitumoral effects against SCC7 and B16F10 tumors, suggesting that HD might not be the main drug for determining therapeutic effects in the combination therapy. However, the cellular internalization of DOX is much higher in B16F10 cells than that in SCC7 cells, implying that DOX has much higher sensitivity to B16F10 than to SCC7. According to the previous reports, IC_{50} values of free DOX against B16F10 and SCC7 cells were 0.41~1.03 μM (29,30) and 2.6 μM (28), respectively, implying that DOX showed more drug resistance against SCC7 than B16F10 cells. Thus, the combination therapy against B16F10 tumors leads to higher cytotoxicity, apoptosis induction *in vitro* and *in vivo*, and finally shows superior antitumoral effects on SCC7. Taking these results into consideration, it can be said that the internalization of anticancer drugs such as DOX against tumor cells may also play a pivotal aspect in determining therapeutic effects in combination therapy. In the present study, it is thought that the main reason for the enhanced therapeutic effects of the combination therapy is because of the increased apoptosis induction *in vitro* and *in vivo* against two tumors, although the antiproliferative and antiangiogenic effects of HD could affect tumor growth inhibition. It will be anticipated that these two drugs can provoke the enhanced therapeutic efficacy by over-expressing the apoptotic proteins such as p53 and p21, and by elevating the ratio of Bax/Bcl-2. The apoptotic mechanism of p53 and p21 proteins will be studied as further work.

In the clinic, combining multiple agents of antiangiogenic and cytotoxic anticancer drugs has been shown to give an additive effect, and these approaches could decrease the chance of resistance to the chemotherapy regimen. In the present study, we contend that HD with low anti-coagulant activity has an advantage over the combination chemotherapy because it has no risk of side effects compared to heparin. It was confirmed that HD is free of associated complications when treated at high doses (from 1 to 20 mg/kg/3 day) for nearly one month. After the treatment of HD for extended time periods, we found that the level of platelets remained in normal (25): this suggests that HD may not induce such side effects as bleeding or thrombocypenia. In addition, other parameters including ALT, AST, creatinine, and BUN were normal, indicating that the liver and renal functions were normal. Therefore, it means that HD is safe in clinical application.

CONCLUSION

We report here that the concurrent treatment of HD and DOX significantly enhances the antitumoral effect of DOX against SCC7 and B16F10 tumors in murine mice models. We found that when HD, which has antiproliferative effect against SCC7 and B16F10 cells, was combined with DOX, cellular toxicity and apoptosis induction were significantly increased in tumor cells. Moreover, we report that the improved antitumoral effects of DOX *in vivo* might be due to the synergistically induced apoptosis.

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REFERENCES

1. R. J. Linhardt, and D. Loganathan. In C. G. Gelelein (ed.), *Biomimetic Polymers*, Plenum, New York, 1990, p. 135.
2. P. S. Damus, M. Hicks, and R. D. Rosenberg. Heparin—a generalized views of its anticoagulant action. *Nature* **246**:355–356 (1973).
3. M. Petitou, J. P. Herault, A. Bernat, J. M. Herbert, and J. C. Lormeau. Synthesis of thrombin-inhibiting heparin mimetics without side effects. *Nature* **398**:417–422 (1999).
4. M. Henja, M. Raderer, and C. C. Zielinski. Inhibition of metastases by anticoagulants. *J. Natl. Cancer Inst.* **91**:22–36 (1999).
5. I. Capila and R. J. Linhardt. Heparin-protein interactions. *Angew. Chem. Int. Ed. Engl.* **41**:391–412 (2002).
6. S. M. Smorenburg and C. J. NoordenVan. The complex effects of heparins on cancer progression and metastasis in experimental studies. *Pharmacol. Rev.* **53**:93–105 (2001).
7. G. Mannori, P. Crottet, O. Cecconi, K. Hanasaki, A. Aruffo, R. M. Nelson, A. Varki, and M. P. Bevilacqua. Differential colon cancer cell adhesion to E-, P-, and L-selectin: role of mucin-type glycoproteins. *Cancer Res.* **55**:4425–4431 (1995).
8. L. Borsig, R. Wong, O. Hynes, N. M. Varki, and A. Varki. Synergistic effects of L- and P-selectin in facilitating tumor metastasis can involve non-mucin ligands and implicate leukocytes as enhancers of metastasis. *Proc. Natl. Acad. Sci. USA* **99**:2193–2198 (2002).
9. L. Borsig. Selectins facilitate carcinoma metastasis and heparin can prevent them. *News Physiol. Sci.* **19**:16–21 (2004).
10. R. J. Ludwig, B. Boehme, M. Podda, R. Henschler, E. Jager, C. Tandi, W.-H. Boehncke, T. M. Zollner, R. Kaufmann, and J. Gille. Endothelial P-selectin as a target of heparin action in experimental melanoma lung metastasis. *Cancer Res.* **64**:2743–2750 (2004).
11. J. R. Guyton, R. D. Rosenberg, A. W. Clowes, and M. J. Karnoysky. Inhibition of rat arterial smooth muscle cell proliferation by heparin. *In vivo* studies with coagulant and non-anticoagulant heparin. *Circ. Res.* **46**:625–634 (1980).
12. H. R. Mason, R. A. Nowak, C. C. Morton, and J. J. Castellot Jr. Heparin inhibits the motility and proliferation of human myometrial and leiomyoma smooth muscle cells. *Am. J. Pathol.* **162**:1895–1904 (2003).
13. N. S. Fedarko, M. Ishihara, and H. E. Conrad. Control of cell division in hepatoma cells by exogenous heparan sulfate proteoglycan. *J. Cell Physiol.* **139**:287–294 (1989).
14. S. S. Karti, E. Ovali, O. Ozgur, M. Yilmaz, M. Sonmez, S. Ratip, and F. Ozdemir. Induction of apoptosis and inhibition of growth of human hepatoma HepG2 cells by heparin. *Hepatogastroenterology* **50**:1864–1866 (2003).
15. J. Manaster, J. Chezar, R. Shurtz-Swirski, G. Shapiro, Y. Tendler, B. Kristal, S. M. Shasha, and S. Sela. Heparin induces apoptosis in human peripheral blood neutrophils. *Br. J. Haematol.* **94**:48–52 (1996).
16. E. Erduran, Y. Tekelioglu, Y. Gedik, and A. Yildiran. Apoptotic effects of heparin on lymphoblasts, neutrophils, and mononuclear cells: results of a preliminary *in vitro* study. *Am. J. Pathol.* **61**:90–93 (1999).
17. H. L. Li, K. H. Ye, H. W. Zhang, Y. R. Luo, X. D. Ren, A. H. Xiong, and R. Situ. Effect of heparin on apoptosis in human nasopharyngeal carcinoma CNE2 cells. *Cell Res.* **11**:311–315 (2001).
18. M. N. Levine, J. Hirsh, J. G. Kelton. Heparin-induced bleeding. In D. A. Lane and U. Lindhal (eds.), *Heparin: Chemical and biological properties, Clinical Applications*, CRC, 1989. pp. 517–531.
19. J. D. Douketis, J. S. Ginsberg, R. F. Burrows, E. K. Duku, C. E. Webber, and P. Brill-Edwards. The effects of long-term heparin therapy during pregnancy on bone density. A prospective matched cohort study. *Thromb. Haemost.* **75**:254–257 (1996).
20. T. Irimura, M. Nakajima, and G. L. Nicolson. Chemically modified heparins as inhibitors of heparan sulfate specific endo-β-glucuronidase (heparanase) of metastatic melanoma cells. *Biochemistry* **25**:5322–5328 (1986).
21. P. E. Thorpe, E. J. Derbyshire, S. P. Andrade, N. Press, P. P. Knowles, S. King, G. J. Watson, Y. C. Yang, and M. Rao-Bette. Heparin-steroid conjugates: new angiogenesis inhibitors with antitumor activity in mice. *Cancer Res.* **53**:3000–3007 (1993).
22. K. Ono, M. Ishihara, K. Ishikawa, Y. Ozeki, H. Deguchi, M. Sato, H. Hashimoto, Y. Saito, H. Yura, A. Kurita, and T. Maehara. Periodate-treated, non-anticoagulant heparin-carrying polystyrene (NAC-HCPS) affects angiogenesis and inhibits subcutaneous induced tumor growth and metastasis to the lung. *Br. J. Cancer* **86**:1803–1812 (2002).
23. C. Y. Pumphrey, A. M. Theus, S. Li, R. S. Parrish, and R. D. Sanderson. Neoglycans, carbodiimide-modified glycosaminoglycans: a new class of anticancer agents that inhibit cancer cell proliferation and induce apoptosis. *Cancer Res.* **62**:3722–3728 (2002).
24. K. Park, K. Kim, I. C. Kwon, S. K. Kim, S. Lee, D. Y. Lee, and Y. Byun. Preparation and characterization of self-assembled nanoparticles of heparin–deoxycholic acid conjugates. *Langmuir* **20**:11726–11731 (2004).
25. K. Park, G. Y. Lee, Y. S. Kim, M. Yu, R. W. Park, I. S. Kim, S. Y. Kim, and Y. Byun. Heparin–DOCA acid chemical conjugate as an anti-cancer drug carrier and its anti-tumor activity. *J. Control. Release* **114**:300–306 (2006).
26. K. Park, Y. S. Kim, G. Y. Lee, J. O. Nam, S. K. Lee, R. W. Park, S. Y. Kim, I. S. Kim, and Y. Byun. Antiangiogenic effect of bile acid acylated heparin derivative. *Pharm. Res.* **24**:176–185 (2007).
27. K. Hida, Y. Hida, and D. N. Amin. Tumor-associated endothelial cells with cytogenetic abnormalities. *Cancer Res.* **64**:8249–8255 (2004).
28. G. Y. Lee, K. Park, J. H. Nam, S. Y. Kim, and Y. Byun. Anti-tumor and anti-metastatic effects of gelatin–doxorubicin and PEGylated gelatin–doxorubicin nanoparticles in SCC7 bearing mice. *J. Drug Target.* **14**:707–716 (2006).
29. R. Tomlinson, J. Heller, S. Brocchini, and R. Duncan. Polyacetal–doxorubicin conjugates designed for pH-dependent degradation. *Bioconj. Chem.* **14**:1096–1106 (2003).
30. F. M. Veronese, O. Schiavon, G. Pasut, R. Mendichi, L. Andersson, A. Tsirk, J. Ford, G. Wu, S. Kneller, J. Davies, and R. Duncan. PEG–Doxorubicin conjugates: influence of polymer structure on drug release, *in vitro* cytotoxicity, biodistribution, and antitumor activity. *Bioconj. Chem.* **16**:775–784 (2005).
31. B. Agarwal, B. Halmos, A. S. Feoktistov, P. Protiva, W. G. Ramey, M. Chen, C. Pothoulakis, J. T. Lamont, and P. R. Holt. Mechanism of lovastatin-induced apoptosis in intestinal epithelial cells. *Carcinogenesis* **23**:521–528 (2002).
32. C. F. Reilly, M. S. Kindy, K. E. Brown, R. D. Rosenberg, and G. E. Sonenshein. Heparin prevents vascular smooth muscle cell progression through the G1 phase of the cell cycle. *J. Biol. Chem.* **264**:6990–6995 (1989).
33. R. D. Rosenberg. Vascular smooth muscle proliferation: basic investigations and new therapeutic approaches. *Thromb. Haemost.* **70**:10–16 (1993).
34. S. J. Busch, G. A. Martin, R. L. Barnhart, M. Mano, A. D. Cardin, and R. L. Jackson. Trans-receptor activity of nuclear glycosaminoglycans on Fos and Jun/AP-1 oncoprotein mediated transcription. *J. Cell Biol.* **116**:31–42 (1992).
35. A. V. Kabanov and V. A. Kabanov. DNA complexes with polycations for the delivery of genetic material into cells. *Bioconj. Chem.* **6**:7–20 (1995).
36. Y. Lee, J. H. Nam, H. C. Shin, and Y. Byun. Conjugation of low-molecular-weight heparin and deoxycholic acid for the development of a new oral anticoagulant agent. *Circulation* **104**:3116–3120 (2001).