Abstract. Background: Heparin is a polysulfated glycosaminoglycan that has been shown to have antiproliferative and apoptotic effects in addition to its anticoagulant effects. Materials and Methods: The present work investigated the effects of unfractioned heparin (UFH) on cell growth and apoptosis in four oral squamous cell carcinoma (SCC) cell lines and the mechanism(s) underlying its actions using MTT assay, Annexin-V-FITC and Western blotting. Results: Treatment with UFH resulted in significant reduction in cell viability and increase in apoptosis in three of the four tested cell lines. Further, such treatment resulted in a significant decrease in phosphorylated AKt, and consequently led to activation of the mitochondrial pathway in heparin-sensitive cells. Moreover, pretreatment with UFH significantly increased the apoptosis induced by cisplatin. Conclusion: These findings indicate that heparin induces apoptosis through suppression of AKt, and suggest a potential utility of heparin for development of less toxic chemotherapy in treatment of oral SCC.

Heparin is a member of the family of polysaccharides, the glycosaminoglycans (GAGs) which are linear carbohydrate polymers and are composed of alternating uronate and hexosamine saccharides (1). The other members of this family include heparan sulfate, chondroitin sulfate and dermatan sulfate. These structurally complex members confer the GAG chains a net negative charge. The property of having highly negative charge allows heparin to interact with a large number of proteins and basic molecules through ionic and hydrogen bonding interactions (2, 3), and thus enables it to have a variety of biological activities other than its anticoagulant function. In 1989, Regelson proposed that polyanionic substances such as heparin, a member of the GAG group, are tumor inhibitors (4). Later studies further demonstrated that heparin affects proliferation, migration and invasion in various cell types, including those derived from epithelial cells (3, 5-8), and induces apoptosis in lymphoblasts (9) and nasopharyngeal cancer cells (10). However, the mechanisms related to these actions of heparin remain unclear.

Studies have shown that heparin may inhibit the phosphorylation of numerous signaling pathways, including protein kinase C (PKC), mitogen activated protein kinase (MAPK) and casein kinase II (CKII) (11-14). CKII is the major multipotential serine-threonine kinase identified in a variety of cell types (15) and its activation has been linked with upregulation of AKt (16, 17). These notions indirectly suggest a possibility that heparin may negatively affect the activation of AKt, a serine/threonine protein kinase that mediates various downstream effects of phosphatidylinositol 3'-kinase (PI3-K) (18) and plays a central role in regulating many biological processes, such as proliferation, apoptosis and cell growth (19, 20).

Oral cancer, consisting mainly of squamous cell carcinoma (SCC), is one of the leading causes of cancer death worldwide (21, 22). Although the disease at operable stage is mostly curable, more than 50% of cases are advanced at presentation and fewer than 30% of them can be cured (23). Chemotherapy is one of the adjuvant or adjunctive treatments for improving overall survival of the advanced cases. However, the generation of drug resistance and hematological cytotoxicity often prevent anticancer drugs from achieving their desired outcome. Development of new strategies to overcome the hurdles in chemotherapy would be beneficial for improvement of clinical outcome in treatment of the patients with advanced oral cancer.

In view of the existing evidence, it was hypothesized that the reported actions of heparin may also be exerted in oral
SCC cells, and hence the effects of unfractioned heparin (UFH) on cell growth and apoptosis in four oral SCC cell lines were investigated. Here, it is demonstrated that treatment with UFH results in significant inhibition of cell growth and induction of apoptosis. It is hereby reported, for the first time, that UFH suppresses the phosphorylation of AKt in heparin-sensitive cells, which was found to be linked to its apoptotic effect. Moreover, it is demonstrated that pretreatment with UFH enhances the cytotoxic effect of cisplatin.

Materials and Methods

Cell lines and culture. Human oral SCC cell lines of HSC-4, KOSCa, KON and HO-N1 were obtained from Cell Bank of Human Science Resources of Japan (CBHSJ). HSC-4 was grown in MEM media (HyClone, Logan, Utah, USA), KOSCa in RPMI 1640 (HyClone), and KON and HO-N1 grown in DEM/12-F. The media were supplemented with 10% fetal bovine serum (FBS) (BioWest, AbCy s.a., France) and 1% (v/v) solution of penicillin 10,000 U/mL and streptomycin 10,000 μg/mL (HyClone). Cells were maintained at 37˚C in an atmosphere containing 5% CO2 and 100% humidity.

MTT assay on cell viability. The inhibitory effect of heparin on cell viability was assessed by MTT assay (24). Briefly, cells were seeded in 100-mm dishes at density of 100 cells/μL and, after 24-h culture in media, cells were detached by trypsinization. The detached cells were seeded in 96 well plates at a density of 5,000 cells per well in 0.1 mL media with vehicle (0.1% dimethyl sulfoxide, DMSO) as control or unfractioned heparin UFH (Sigma-Aldrich, St. Louis, MO, USA) at concentrations of 5 U/mL, 20 U/mL and 80 U/mL. After 12, 24 and 48 h incubations, a 10 μL aliquot of MTT solution was added to each well and then incubated for another 4 h. Finally, isopropyl was added as a solvent to each well and, after shaking the plate for 2 min, the absorbance in each plate was measured at a wavelength of 570 nm with a BioRad Model 450 microplate reader (Hercules, CA, USA). Three wells were used for each concentration and the experiment was replicated 4 times.

Apoptosis assay. Quantitation of apoptosis was performed by flow cytometry based on Annexin-V FITC and propidium iodide (PI) double staining. Briefly, cells were seeded in 60-mm dishes at a density of 100 cells/μL and cultured for 24-h, after which the cells were treated with vehicle (0.1% dimethyl sulfoxide, DMSO) as control, unfractioned heparin UFH (Sigma-Aldrich) alone, cisplatin (Nippon Kayaku, Japan) alone, a combination of cisplatin plus pretreatment of UFH, or Ly294002 of AKt/PI3-I inhibitor (25). UFH or cisplatin treatment was performed for 12, 24 and 48 h, respectively at concentrations of 5 U/mL, 20 U/mL and 80 U/mL. For the combination treatment, cisplatin treatment was performed after pretreatment with UFH at concentration of 20 U/mL for 24 h. Treatment with Ly294002 at concentrations of 10 μM, 20 μM and 40 μM was performed for 24 h. After the above mentioned treatments, cells were detached by trypsinization. Detached cells were washed with phosphate-buffered saline (PBS) and re-suspended in 100 μL ice-cold binding buffer (Annexin V–FITC Kit; Immunotech, Marseille, France) containing 1 μL of Annexin V–fluorescein isothiocyanate (FITC) stock and 5 μL of 20 μg/μL PI. After incubation for 15 min on ice in darkness, the samples were examined by flow cytometry using a FACScan (Beckton Dickinson, San Jose, CA, USA), acquiring 10,000 events. Data analysis was performed using Cell Quest software (Beckton Dickinson). The cells in the subpopulations labeled by the following two staining patterns were considered as apoptotic cells: first, staining of Annexin-V-FITC (+)/PI (−) indicating early apoptotic cells; second, staining of Annexin-V-FITC (+)/PI (+) indicating late apoptotic/necrotic cells.

Cell fractionation. HSC-4 and HO-N1 cells treated with vehicle (0.1% dimethyl sulfoxide, DMSO) as control, UFH or Ly294002 were scraped and washed with PBS on ice. The whole cells were lysed with lysis buffer (10mM Tris-HCl (pH7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 0.5% Nonidet P-40) containing a protease inhibitor mixture (Roche Diagnostics, Penzberg, Germany). The lysates were sonicated on ice and centrifuged at 15,000 xg for 10 min at 4˚C and the resulting supernatants were collected as the whole cell lysates.

Mitochondria and cytosol fractionation was performed using Mitochondria/Cytosol Fractionation Kit (BioVision, Mountain View, CA, USA), which contains Mitochondria Extraction Buffer, Cytosol Extraction Buffer, DTT and Protease Inhibitor Cocktail. The mitochondria and cytosol extraction buffer mix with DTT and protease inhibitor cocktail were prepared according to the protocol of BioVision. Briefly, the scraped cells were collected by centrifugation at 600 xg for 5 min at 4˚C, washed with ice-cold PBS, and centrifuged at 600 xg for 5 min at 4˚C. Cells were re-suspended with 1.0 mL of 1X Cytosol Extraction Buffer Mix containing DTT and protease inhibitor cocktail, and incubated on ice for 10 min. Next, cells were homogenized by 30-50 passes in a dounce tissue grinder on ice. The homogenates were transferred to a 1.5-mL microcentrifuge tube and centrifuged at 700 xg (~3,000 rpm) for 10 min at 4˚C. The supernatant fraction was carefully collected and the pellet was discharged. The collected supernatant was transferred to a fresh 1.5-mL tube, and centrifuged at 10,000 xg (~13,000 rpm) for 30 min at 4˚C. The resulting supernatant was collected as the cytosolic fraction. Further, the pellet containing the enriched mitochondria was resuspended with 100 μL of the Mitochondrial Extraction Buffer Mix containing DTT and protease inhibitor, vortexed for 10 seconds, and saved as the mitochondrial fraction.

Western blot analysis. Western blot analysis was used to probe AKt; phosphorylated AKt 1 (Ser473) and AKt 2 (Thr308); Bad; phosphorylated Bad; Bcl-xL; cytochrome c; procaspase-9 and β-actin were used as controls. All of the primary antibodies used for immunoblotting were purchased from Cell Signaling Technology. Briefly, the whole cell lysates and cytosolic and mitochondrial fractions were assayed for protein concentration using the Bio-Rad Dye Binding Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of the protein from each sample were diluted in 2x sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, electrophoresed on 10-20% SDS-polyacrylamide gels. The protein was then transferred to PVDF membrane by electro-blotting. After washing with PBS and blocking in PBS and 5% non-fat milk, the membrane was incubated with mouse monoclonal or rabbit polyclonal primary antibodies at dilution of 1:1,000 in PBS and 2% non-fat milk overnight at 4˚C, followed by incubation with goat anti-mouse or rabbit secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology) at room temperature for 1 h. Finally, the blot was visualized by use of enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK). Bands on Western blots were analyzed densitometrically using Scion Image software (version 4.0.2; Scion Corp., Frederick, MD, USA).
Results

Effect of heparin on cell viability and apoptosis. The antiproliferative and apoptotic effects of heparin have been documented for numerous cell types (9, 10, 26). To investigate these actions of heparin on oral SCC cells, the four oral SCC cell lines of HSC-4, KOSC-2, KON and HO-N1 were used for analysis. After treatment with UFH at various concentrations, cell viability at different time points was assessed by the MTT assay. The treatment with UFH at concentrations of 20 U/mL and 80 U/mL resulted in significant reduction of cell viability in the three cell lines HSC, KOSC and KON at 12, 24 and 48 h incubation, with a peak at the 24 h time point (Figure 1-A). Consistently, in these cell lines the same treatment induced a significant increase in apoptosis, which peaked at 24 h (Figure 1-B).

Figure 1. Inhibitory effect of UFH on cell growth and its apoptotic effect in HSC-4, KOSC-2, KON and HO-N1. (A) Reduction of cell viability. Cells were treated with UFH or 0.1% DMSO as control. Cell viability was assessed by MTT assay. In each experiment, cell viability in control was designated as 100%. Data represent the mean±SD of reduction in cell viability of four independent experiments. Statistical significance relative to control is denoted by *(p<0.05, Student’s t-test). (B) Induction of apoptosis. After treatment with UFH at the indicated concentrations and time, cells were collected and stained with Annexin-V-FITC (FITC) and propidium iodide (PI). Induction of apoptosis was assessed by flow cytometry. Data represent the mean±SD of the percentage of the apoptotic cells in both early and late stages in three independent experiments. Statistical significance relative to control is denoted by *(p<0.05, Student’s t-test).
However, for HO-N1 cells, such treatment failed to result in a distinct reduction of cell viability and apoptotic induction. Therefore, HSC-4, KOSC-2 and KON were defined as heparin-sensitive, and HO-N1 as heparin-insensitive.

The data shown in Figure 1 A-B indicate that the significant effect of UFH on cell viability and apoptosis may be achieved from the concentration of 20 U/mL, and that a further increase of the dose to 80 U/mL did not significantly enhance the effect of UFH. Because 20 U/mL is a clinically available concentration for UFH administration, this concentration was selected for further assays on elucidation of the pathways mediating heparin-induced apoptosis.

**Inhibitory effect of heparin on phosphorylation of AKt and Bad.** Heparin has been shown to exert an inhibitory effect on the phosphorylation of numerous signaling pathways (11-14), one of which, CKII, is associated with the up-regulation of AKt (16, 17). Studies have demonstrated that AKt is constitutively activated in carcinomas of the oral cavity and head and neck, and that suppression of AKt by its inhibitor promotes apoptotic induction in this tumor type (27, 28). These notions led to the hypothesis that the heparin-induced apoptosis in this assay is mediated through suppression of AKt. Hence, the heparin-sensitive cell line HSC-4 and the heparin-insensitive cell line HO-N1 were used for elucidation of this presumption. The effect of heparin on Bad, a pro-apoptotic factor of BCL-2 family, which is phosphorylated by AKt and regulates the activity of antiapoptotic factor Bcl-xL was also examined. As shown in Figure 2, both cell lines expressed phosphorylated (p)AKt (Ser473 and Thr308) and phosphorylated (p)Bad in control conditions. Treatment of the heparin-sensitive cell line HSC-4 with UFH at 20 U/mL resulted in a significant decrease in pAKt (Ser473 and Thr308) and pBad at 12-24 h. For the heparin-insensitive cell line HO-N1, UFH treatment did not result in any obvious decrease in pAKt (Ser473 and Thr308) and pBad. These findings reveal a constitutive activation of pAKt in oral SCC cells and suggest that heparin exerts a suppressive effect on the phosphorylation of AKt in sensitive cells, leading to the inactivation of Bad.

**Effect of heparin on apoptosis-related mediators.** Akt is phosphorylated following PI3K activation and subsequently phosphorylates a number of proteins with pro-apoptotic activities, such as Bad, leading to their inactivation (29, 30). Phosphorylation of Bad displaces it from Bcl-xL, an anti-apoptotic factor of the Bcl-2 family, and thereby allows Bcl-xL to play its anti-apoptotic role (31, 32). Inhibition of Bcl-xL by dephosphorylation of Bad via inactivation of AKt or down-regulation of Bcl-xL directly by inhibitors of AKt/IP3-K, has been shown to be associated with progression of apoptosis (29, 33, 34). Therefore, it was deduced that the inactivation of the AKt-Bad pathway by UFH treatment may lead to inhibition of the anti-apoptotic activity of Bcl-xL and thereby activation of the mitochondria caspase cascade in the progress of apoptosis. In addition, the inhibition of AKt by its inhibitors has been demonstrated to cause direct decrease in Bcl-xL in several in vitro studies (35, 36). To investigate the putative cellular
responses resulting from the suppression of the AKt-Bad pathway by heparin, the changes in the Bcl-xL protein level in the mitochondria, and the cleavage of caspase-9 in HSC-4 and HO-N1 cells treated with UFH were examined using Western blot analysis. Treatment with UFH at 20 U/mL resulted in a significant decrease in Bcl-xL, an increase in cytochrome c, and an increase in cleaved caspase-9 in sensitive cells of HSC-4 but not in insensitive cells of HO-N1 (Figure 3). The changes of these cellular proteins appeared after 6 h and peaked at 24 h. Identification of the release of cytochrome c from mitochondria into the cytosol and the cleaved caspase-9 indicates that activation of the mitochondrial signal pathway mediates the heparin-induced apoptosis.

Effect of AKt/PI3-K inhibitor on heparin-insensitive cell line. To further confirm if the suppression of AKt is the early cellular event that results in sequential changes in protein levels of downstream apoptotic signal mediators, heparin-insensitive cells of HO-N1 were treated with Ly294002, a well known AKt/PI3-K inhibitor that specifically suppresses activation of AKt (25). Similarly to the action of heparin on the sensitive cells, treatment with Ly294002 at a concentration of 20 μM at the time of 6-24 h resulted in a decrease in pAKt (Ser473 and Thr308) and pBad, a decrease in Bcl-xL in the mitochondrial fraction, and induction of cleaved caspase-9 in the heparin-insensitive cells (Figure 4). Further, Ly294002 treatment also resulted in a significant increase of apoptosis from the dose concentration of 20 μM (Figure 5). These results indirectly prove that the suppression of AKt by heparin is associated with its apoptotic effect in sensitive cells.

Enhancing effect of heparin on cisplatin cytotoxicity. Recent studies have demonstrated that up-regulation of AKt is associated with cisplatin resistance (37, 38), and that down-regulation of AKt by its inhibitor or siRNA can increase drug sensitivity and reverse drug resistance (35, 39). The presented data showing an apoptotic effect of heparin on oral SCC cells through inhibition of AKt lead to the further examination of the possibility of utilizing heparin to sensitize tumor cells to the cytotoxicity of cisplatin. The induction of apoptosis was compared in the four cell lines treated with cisplatin alone or a combination of cisplatin plus pretreatment of UFH. As shown in Figure 6, compared to the cisplatin-only treatment, the combined treatment resulted in a significant increase of apoptotic induction in HSC-4 (A), KOSC-2 (B) and KON (C), but not in HO-N1 (D).
Discussion

In the present study, it is demonstrated that treatment with unfractioned heparin (UFH) resulted in a significant inhibition of cell growth and induction of apoptosis in three of the four tested oral SCC cell lines, HSC-4, KOSC-2 and KON. Further investigation on the mechanism(s) underlying the apoptotic effect of heparin demonstrates that a) oral SCC cells exhibited a constitutively phosphorylated AKT; b) treatment of sensitive cell line HSC-4 with heparin resulted in suppression of AKT followed by inactivation of Bad, downregulation of Bcl-xL from mitochondria, release of cytochrome c from mitochondria into the cytosol and cleavage of caspase-9; c) for heparin-insensitive cell line of HO-N1, instead of heparin, treatment with Ly294002 of AKT/PI3K inhibitor resulted in a series of cellular events.

Figure 4. Effect of Ly294002 on phosphorylation of AKT and Bad (A), and protein level of Bcl-xL of mitochondria and activation of caspase-9 in HO-N1 cells (B). Cells were treated with Ly294002 at 20 μM, and at the indicated periods protein expressions in whole cell lysates or mitochondrial fraction were analyzed by Western blotting. β-actin was used as loading control. Intensity of protein bands in (A) and (B) was quantified by densitometry. The densities of the protein band in control conditions were designated as 1, and the levels of the remaining samples were shown as fold of the control in (C), (D) and (E). Data are representative of three similar experiments.
been shown to be constitutively active in the cancers of oral cavity and head and neck (27, 28). On the other hand, studies on the mechanisms involved in cisplatin resistance have established that up-regulation of AKt is one of the main cellular events responsible for development of this drug resistance (37, 38). One recent report demonstrated that the down-regulation of AKt 1 by use of AKt 1 siRNA significantly down-regulates the expression of Bcl-2 and up-regulates the expression of Bax in gastric cancer cells, consequently leading to enhancement of sensitivity to several chemotherapy drugs (39). These existing data encouraged the hypothesis that there exists constitutive activation of AKt in oral SCC cells, and that heparin may suppress AKt and thus possess the activity of inducing apoptosis and sensitizing tumor cells to cisplatin. The results verified the presumptions by showing that: 1) pAKt (Ser473 and Thr308) was identified in both the heparin-sensitive cell line HSC-4 and the heparin-insensitive cell line HO-N-1, 2) treatment of the sensitive cells with UFH resulted in a significant decrease in the level of (p)AKt (Ser473 and Thr308) at the time of 12-24 h, which was identical with the time apoptotic induction reached a peak, c) for the heparin-insensitive cell line HO-N1, treatment with Ly294002 of AKt/PI3K inhibitor resulted in similar decrease in the pAKt level. These findings suggest that the constitutively activated AKt in oral SCC cells can be the target of heparin or PI3K/AKT inhibitor, and that the suppression of phosphorylation of AKt by heparin mediates its apoptotic induction.

Phosphorylation of Bad by active AKt dislocates it from Bcl-xL, a member of bcl-2 family, and the dislocated Bcl-xL can play its anti-apoptotic role (31, 32). Inactivation of AKt by the stimuli of triggering apoptotic induction causes dephosphorylation of Bad and promotes its binding to Bcl-xL (33, 42), consequently blocking the inhibitory effect of Bcl-xL on mitochondrial apoptotic cascade involving mitochondria permeability transition (MPT), cytochrome c release from mitochondria into cytosol, caspase-9 activation, and execution of caspases activation (34, 43). To test if such a pathway is involved in heparin-induced apoptosis, the effect of heparin on a sequence of changes in apoptosis-related mediators, including Bad, Bcl-xL, cytochrome c and caspase-9 was examined. It was found that treatment of heparin-sensitive cells of HSC-4 with UFH results in a significant decrease in pBad of mitochondria and an increase in cytochrome c of cytosol and induces cleaved caspase-9 at the same time as the decrease of pAKt is observed. However, these events were not identified in heparin-insensitive cells of HO-N1 that exhibited no decrease in pAKt upon to UFH treatment. These findings suggested a possible association between suppression of AKt and apoptotic effect of heparin in oral SCC cells. In addition, a distinct decrease of Bcl-xL at the time of 12-24 h after UFH treatment was also observed, which is in agreement with several in vitro studies where suppression of AKt phosphorylation by specific inhibitors was shown to be
associated with down-regulation of Bcl-xL (33). It is possible that heparin may directly inhibit Bcl-xL to promote apoptosis. Further, to obtain the evidence indicating that inactivation of AKt mediates induction of apoptosis in oral SCC cells, the heparin-insensitive cell line HO-N1 was treated with AKt/PI3K inhibitor, and the results obtained were similar to those in HSC-4 triggered by UFH, as shown by a sequence of apoptosis-related cellular responses. The inhibitor treatment also resulted in a significant increase of apoptosis. These findings suggest the existence of a mechanism of suppression of AKt in oral SCC cells, by which heparin or AKt/PI3K inhibitor induces apoptosis.

Cisplatin is an effective DNA-damaging anticancer agent used for the treatment of various types of human cancers. In the last decades, it has been shown to be active for carcinomas in the oral cavity and head and neck (44, 45). However, the hematological cytotoxicity or the acquisition of drug resistance often prevents its treatment from reaching the desirable therapeutic goals. Therefore, it is of significance that in this assay UFH was shown to significantly increase the apoptosis induced by cisplatin, and that such effect could been achieved at a clinically available concentration of 20 U/mL. Ongoing work is being performed on the examination of the enhancing effect of heparin on several chemotherapy drugs in an animal model.

In conclusion, the inhibitory effect of heparin on cell viability and its apoptotic effect in oral SCC cells have been demonstrated. It has been reported, for the first time, that heparin suppresses activation of AKt and that such a suppressive effect mediates its apoptotic effect. Further, the advantage that these effects were achieved at clinically available concentrations suggests a possibility to apply such a setting, heparin pretreatment plus cisplatin, into clinical practice sooner than other chemically synthesized AKt inhibitors.

References


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