Combination of nadroparin with radiotherapy results in powerful synergistic antitumor effects in lung adenocarcinoma A549 cells

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Abstract. Low-molecular-weight heparins (LMWHs), which are commonly used in venous thromboprophylaxis and treatment, have recently been reported to have effects on cancer metastasis in pre-clinical research studies. This study was planned to define the synergistic antitumor effects of nadroparin (a kind of LMWH) combined with radiotherapy in A549 cells. Six experimental groups were set up in our study according to the different treatment: control group; irradiation (IR) group; low dose of nadroparin group (LMWH50, L50); high dose of nadroparin group (LMWH100, L100); LMWH50+IR group; LMWH100+IR group. The viability of A549 cells was assessed by Cell Counting Kit-8 (CCK-8) assay. The apoptosis of tumor cells was analyzed by flow cytometry (FCM) after treatment. The concentration of transforming growth factor-β1 (TGF-β1) in the culture supernatants was measured by enzyme-linked immunosorbent assay (ELISA). The migration and invasion of the A549 cells were tested by the Transwell chamber assay. The expression of survivin, CD147 and matrix metalloproteinase-2 (MMP-2) was analyzed by western blotting.

CCK-8 assay showed that irradiation or nadroparin alone slightly inhibited the cell viability while the combined treatments significantly inhibited the cell viability in a dose- and time-dependent manner. The apoptosis rate showed greater improvement dose- and time-dependently in the groups receiving combination therapy of nadroparin and irradiation than the control group or the group receiving nadroparin alone. The Transwell chamber assay showed that nadroparin not only significantly suppressed the migration and invasion of A549 cells but also inhibited the enhanced ability of migration and invasion induced by X-ray irradiation. Western blotting showed that nadroparin inhibited the upregulated effects of survivin and MMP-2 expression induced by irradiation in the combined treatment groups in a dose- and time-dependent manner. Moreover, the expression level of CD147 was the lowest in the combined treatment groups. This study identified that combination of nadroparin and irradiation had a strong synergistic antitumor effect in a dose- and time-related manner in vitro, which was reflected in the inhibition of cell viability, invasion and metastasis, promotion of apoptosis, inhibited secretion level of TGF-β1 and downregulation of CD147, MMP-2 and survivin expression.

Introduction

Lung cancer has one of the highest morbidity and mortality rates worldwide. The majority of lung cancers are diagnosed at late stages, with locally advanced disease and distant metastases, and its long-term survival remains desperately poor. Radiotherapy is one of the most important therapies in these patients while recurrence and metastasis, signs of malignancy and the main cause of death in cancer patients (1), and the risk of tumor radio-resistance limit the effect of radiotherapy. Many studies have also found that radiotherapy plays a role in stimulating tumor cell growth and promoting invasiveness of tumor cells in addition to a number of common adverse reactions. Thus, looking for the effective combination therapy to enhance the antitumor effect is still urgent.

Low-molecular-weight heparins (LMWHs), obtained by the methods of enzymatic hydrolysis or chemical degradation from unfractionated heparin (UFH), relative molecular weight 3-9 kDa, was approved by the FDA in 1998 for anticoagulant therapy and has been administrated as effective anticoagulants in the prevention and treatment of thrombosis safely for many years (2), along with having potential anticancer effects and improving survival in cancer patients (3,4). Furthermore, recent studies have showed that LMWHs can directly induce the inhibition of the invasion and metastasis of the cancer cells, change the cell cycle, increase the sensitivity of chemotherapy and reduce the extent of radiation-induced liver injury (5-8). Whether it has synergistic antitumor effects to radiotherapy has rarely been reported. On the basis of present studies, we used two doses of nadroparin, a kind of LMWH, combined with X-ray irradiation to treat A549 cells in order to assess the

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relevant interactions between nadroparin and radiotherapy in this study.

**Materials and methods**

**Main instruments and reagents.** A549 cell line used in this study was kindly supplied by the Shanghai Institute of Life Science, Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 medium (Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (FBS). Cultures were maintained at 37˚C in a humidified atmosphere containing 5% CO₂. Nadroparin (Fraxiparina®; GlaxoSmithKline, London, UK) was obtained as standard drug formulations. The Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) was used to detect cell proliferation and activity. Levels of transforming growth factor-β (TGF-β) in the culture supernatants were measured using a standard kit (Enzyme-linked immunosorbent assay (ELISA) kit) from R&D Systems, Inc. (Minneapolis, MN, USA). Annexin-FITC and propidium iodide (PI), and incubated in the dark at room temperature for 15 min. Cells were then analysed in a FACSCalibur flow cytometer (Becton-Dickinson) to differentiate apoptotic cells (Annexin V-positive and PI-negative, lower right quadrant) from necrotic cells (Annexin V/PI-positive, upper right quadrant). Fifteen thousand events were recorded for each treatment group.

**Measurement of TGF-β1 release.** The cell culture supernatants were collected at 24 and 48 h after radiation. The culture supernatants were separated by centrifugation and stored at -80˚C. The concentration of TGF-β1 was measured simultaneously using an ELISA kit (R&D Systems, Inc.).

**Determination of migration and invasion.** About 2x10⁵ cells/well were seeded to six-well plates and treated exactly as described above. The cells were harvested and centrifuged at 24 h after radiation and maintained in serum-free RPMI-1640 for 12 h. Cell invasion and migration were determined with or without Matrigel-coated Transwell chambers. Cells (1x10⁴) were removed for migration and 2x10⁴ cells for invasion of these treated cells were placed into the upper compartment with 100 µl serum-free RPMI-1640, and the lower compartment was filled with RPMI-1640 containing 15% FBS. The chamber was then cultivated in 5% CO₂ at 37˚C for 24 h to detect the cell migration and invasion. The Matrigel and cells in the upper chamber were removed, and the attached cells in the lower section were stained with 0.1% crystal violet. These cells were counted in five high-power microscope fields of vision and photographed.

**Protein extraction and western blotting.** Cells (2x10⁵) were seeded in six-well plates and treated as described above. At the end of the treatment period (24 and 48 h after radiation), cells were washed three times in ice-cold PBS and lysed for at least 30 min on ice in the cold lysis buffer with 1 mM phenylmethanesulfonyl fluoride (PMSF). Protein concentrations were measured using the Coomassie Blue Fast staining solution (Beyotime, Shanghai, China). Proteins (50 µg) in each group was separated on 10-12% SDS-PAGE and transferred to a PVDF membrane (Millipore Corp., Billerica, MA, USA) at 80 V for 90 min. Membranes were blocked by TBS/T containing 5% skim milk for 3 h, and incubated with the CD147, matrix metalloproteinase-2 (MMP-2) and survivin antibody (1:500-1:1,000 dilution) overnight at 4˚C. β-actin antibody (1:10,000 dilution) was used as an endogenous reference for quantification. Then the membranes were incubated with the secondary antibodies (1:4,000 dilution) at room temperature for 1 h after three washes in TBS/T. After several washes with TBS/T, the blots were detected using Immobilon™ Western Chemiluminescent HRP substrate (Millipore Corp.) and quantified using Tanon-4500 Gel Imaging System with GIS ID Analysis Software v4.1.5 (Tanon Science & Technology Co., Ltd., Shanghai, China).

**Statistical analysis.** Statistical analysis was performed using the SPSS software version 20.0. Data were expressed as means ± standard error (SE). Student’s t-test was used to test the differences between groups. Differences resulting in p<0.05 were considered to be statistically significant. All data are reported from three independent experiments.
Results

Effects on cell viability. This study examined the cell viability of A549 cells treated with two doses of nadroparin and X-ray radiation at different time-points, respectively. Significant differences were observed in the treated groups compared to the control group ($p<0.05$, $t=9.492, 12.889, 6.753, 8.153$ and $14.704$ at $24$ h, $t=20.413, 7.990, 17.770, 44.692$ and $29.615$ at $48$ h). A difference from X-ray irradiation group ($p<0.05$, $t=2.883$ and $7.256$ at $24$ h, $t=3.639$ and $6.214$ at $48$ h); a difference from $L_{50}$ ($p<0.05$, $t=3.483, 5.083$ and $10.643$ at $24$ h, $t=11.152, 19.150$ and $18.200$ at $48$ h, $p<0.05$); a difference from $L_{100}$ ($p<0.05$, $t=4.486$ and $9.473$ at $24$ h, $t=5.264, 8.095$ and $9.826$ at $48$ h); a difference from $X+L_{50}$ ($p<0.05$, $t=2.886$ at $24$ h, $t=4.050$ at $48$ h).

Cell apoptosis. LWMHs induced apoptosis of tumor cells in vitro and in vivo (9,10). Our study examined whether nadroparin had synergistic contribution to tumor cell apoptosis when combined with X-ray irradiation. As shown in Figs. 2 and 3, each treatment remarkably induced A549 cell apoptosis dose- and time-dependently compared to the control group. The promotion of apoptosis induced by X-ray irradiation alone was more significant than by different dose of nadroparin alone ($p<0.05$). Furthermore, the cell viability was significantly inhibited in the high dose of nadroparin combined with X-ray irradiation group compared to the other groups ($p<0.05$).

Release of TGF-β1. The concentrations of TGF-β1 in the cell supernatants at the time-points (24 and 48 h) after irradiation were compared among each group in Fig. 4. We found that the TGF-β1 levels were increased with the prolonging of time in the control group, X-ray irradiation group and the $L_{50}$ group, and the increase of the control group was the most obvious. On the contrary, with the prolonging of time, the TGF-β1
levels were significantly decreased in the L100 group and two combined treatment groups. The concentrations of TGF-β1 in X+L100 group were 71.88±5.87 and 37.35±2.92 pg/ml, respectively, at 24 and 48 h after treatment, which was the lowest among the experimental groups (p<0.05).

Cell migration and invasion. Cell migration and invasion are critical for the spreading of cancer and the formation of metastasis in vivo. As shown in Fig. 5, X-ray irradiation alone resulted in a significant increase in A549 cell migration while less migration was observed in the nadroparin alone groups in a dose-dependent manner. Furthermore, nadroparin inhibited the increase in A549 cell migration induced by X-ray irradiation in the combined treatment groups. Additionally, as shown in Fig. 6, similar results were seen in A549 cell invasion. Our result showed that X-ray irradiation promoted the ability of invasion and migration in A549 cells while nadroparin inhibited this side effect dose-dependently.

Expression levels of CD147, MMP-2 and survivin. We examined the protein expression levels of survivin, CD147 and MMP-2 using western blotting in our study to investigate the related molecular mechanism of the antitumor effect associated with nadroparin combined with X-ray radiation on A549 cells. As shown in Fig. 7, the expression levels of CD147 in A549 cells were inhibited by nadroparin in a dose- and time-dependent manner while no significant change of CD147 expression was observed in the X-ray irradiation group. Furthermore, the expression levels of CD147 were the lowest in the combined treatment groups and L100 group at the time of 48 h after treatment (p<0.05). As shown in Fig. 8, at the time of 24 h after treatment, the expression levels of MMP-2 were upregulated in the X-ray irradiation group while downregulated in the groups treated with nadroparin alone (p<0.05). Interestingly, we observed that the upregulated effects induced by radiation were inhibited by nadroparin in the combined treatment groups (p<0.05). Similar tendency of survivin expressions was observed at the time of 24 h after treatment, which is shown in Fig. 9. On the other hand, at the time of 48 h after treatment, the expression levels of MMP-2 were inhibited in the treated groups compared to the control group and the expression levels of survivin were downregulated in the treated groups compared to the control group while the down regulation effect induced by X-ray irradiation alone or the combined treatment was more significant than nadroparin alone (p<0.05).

Discussion
Radiotherapy plays an important role in the treatment of advanced lung cancer. However, the therapeutic efficacy is compromised due to the resistance to X-rays, tumor recurrence and metastasis. In addition, radiation has been shown
to stimulate the tumor cell growth and promote invasiveness of different types of tumor cells in vitro by the upregulation of secreted proteases, such as MMPs and plasminogen activators (11-14). Other researchers have made similar findings in vivo. For instance, Camphausen et al (15) have reported that radiation therapy to a primary tumor accelerates metastatic growth in the Lewis lung cancer mouse model. So it is particularly important to find the effective combination therapy in order to get the best therapeutic efficacy in tumor treatment. LMWH has been reported effective for the treatment of metastasis to some extent, the mechanisms are proposed as anti-coagulation, inhibition of heparanase, selectins, adhesion, angiogenesis mediated by the tumor cells, and the effects on cell cycle and apoptosis (6,16,17). Furthermore, clinical studies have suggested that LMWHs improve life expectancy of lung cancer patients (18-20). So, based on the above, we undertook this experiment to find out the therapeutic effect of nadroparin combined with radiation, and if so, whether there was a dose and time-response relationship for nadroparin also could be analyzed.

Results of our study showed that nadroparin and X-ray irradiation have synergistic antitumor effect in vitro. Nadroparin or X-ray irradiation alone could slightly inhibit the cell viability and enhance the cell apoptosis of A549 cells. The therapeutic effect of X-ray irradiation alone was more significant than that of nadroparin alone. Furthermore, the antitumor effect was
CD147, a member of the immunoglobulin superfamily, is overexpressed in a number of epithelial cell-derived carcinomas and is associated with tumor development and metastasis by activating the fibroblasts producing MMPs (23,24). Wu et al (25) found that CD147 induced resistance to ionizing radiation in hepatocellular carcinoma cells in vitro and in vivo. In our study, we found that nadroparin alone could inhibit the expression of CD147 and MMP-2 in a dose- and time-dependent manner. The expression levels of CD147 and MMP-2 were significantly lower in the combined treatment groups than the control group or X-ray irradiation alone could inhibit the expression of CD147 and MMP-2 in a dose-dependent manner. Based on the results above, we can conclude that the antitumor effect of nadroparin combined with X-ray irradiation may partially relate to the inhibition of TGF-β1 secreted by A549 cells.

CD147, a member of the immunoglobulin superfamily, is overexpressed in a number of epithelial cell-derived carcinomas and is associated with tumor development and metastasis by activating the fibroblasts producing MMPs (23,24). Wu et al (25) found that CD147 induced resistance to ionizing radiation in hepatocellular carcinoma cells in vitro and in vivo. In our study, we found that nadroparin alone could inhibit the expression of CD147 and MMP-2 in a dose- and time-dependent manner. The expression levels of CD147 and MMP-2 were significantly lower in the combined treatment groups than the control group or X-ray irradiation group at the time of 24 and 48 h after treatment. Survivin, the smallest member of the inhibitor of apoptosis protein (IAP) family, encoded by a single gene located on the human 17q25 chromosome with a molecular mass of 16.5 kDa, was first isolated by Ambrosini et al (26) at Yale University. The biological functions of survivin are quite complex, including inhibiting tumor cell apoptosis (27,28), promoting cell proliferation, participating in the regulation of cell cycle (29-31) and promoting blood vessel formation (32), which plays an important role in the development of cancer. Recently, increasing numbers of reports have shown that survivin is an independent prognostic factor for tumor therapy (33,34). It has also showed that survivin overexpression has been correlated with elevated resistance to radiotherapy and chemotherapy in recent studies (35,36). Investigations have reported that ionizing radiation at doses of 1 to 8 Gy is known to significantly elevate survivin levels in malignant cells (37-39). Therefore, blocking tumor cell survivin function or inhibiting its expression may inhibit cell apoptosis and proliferation and enhance its sensitivity to radiotherapy and chemotherapy. In our study, we found that nadroparin alone could inhibit the expression of survivin, which was dose-dependent, but not time-dependent. The expression of survivin was significantly upregulated by X-ray irradiation at the time of 24 h after treatment and this effect was inhibited by nadroparin in a dose-dependent manner. Based on the results above, we can conclude that the expression of CD147, MMP-2 and survivin was effectively and enduringly inhibited by nadroparin combined with X-ray irradiation in the whole process of the treatment. This result suggested that the antitumor effect of nadroparin combined with X-ray irradiation may partially relate to the inhibited expressions of CD147, MMP-2 and survivin in A549 cells.

Taken together, addition of nadroparin to combination radiotherapy resulted in a powerful synergistic antitumor effect in lung adenocarcinoma A549 cells. The mechanism may be associated with the induction of cell apoptosis, reduction of TGF-β1 level, inhibition of cell invasion and migration and downregulated expression of CD147, MMP-2 and survivin. Importantly, these results revealed that nadroparin could inhibit the promotion of cell migration and invasion induced by radiotherapy in a dose-dependent manner. In brief, nadroparin combined with radiotherapy exerted a synergistic antitumor function, which may provide a novel strategy for cancer treatment. However, further investigations are required due to the complexity of structure and function of different LMWHs.

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