Targeting platelet/endothelial cell adhesion molecule 1 enhances cisplatin sensitivity of human nasopharyngeal carcinoma cells exposed to ionizing radiation

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ABSTRACT

Background: Cancer cells develop multidrug resistance after receiving fractionated ionizing radiation. However, the mechanisms underlying this phenomenon remain unknown. This study aimed to investigate the role of platelet/endothelial cell adhesion molecule 1 (PECAM-1), which was induced by ionizing radiation, in overcoming cisplatin resistance of nasopharyngeal carcinoma (NPC) cells. Materials and Methods: Human NPC cell line CNE1 was subjected to fractionated ionizing radiation to obtain a subline with the phenotype of multidrug resistance (designated as CNE1/R). PECAM-1 gene expression in CNE1/R cells was knocked down by stable transfection of pSilencer plasmid carrying specific small hairpin RNA. The transcripts of PECAM-1 and multidrug resistance gene 1 (MDR1) were analyzed by reverse transcription–polymerase chain reaction, and their encoding proteins were detected by Western blot analysis. The in-vitro viability of tumor cells was examined with MTT assay and flow cytometry analysis. The tumor growth in xenograft mice was determined by measuring tumor weights. Results: The transcript and protein levels of PECAM-1 and MDR1 were concomitantly upregulated in CNE1 cells subjected to ionizing radiation. The inhibition of PECAM-1 expression with small hairpin RNA reduced the levels of MDR1 transcript and its encoding protein, P-glycoprotein. Furthermore, targeting PECAM-1 not only enhanced the sensitivity of irradiated CNE1 cells to cisplatin-mediated cell cytotoxicity in-vitro but also resulted in tumor regression in-vivo. Conclusions: An increased PECAM-1 level in CNE1 cancer cells subjected to ionizing radiation contributed to cisplatin resistance via the upregulation of MDR1 expression. Thus, targeting PECAM-1 might help overcome drug resistance induced by ionizing radiation in CNE1 NPC cells.

Keywords: Drug resistance, ionizing radiation, MDR1, nasopharyngeal carcinoma, PECAM-1.

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a special type of head and neck cancer with high incidence in Southeast Asia and China. Early diagnosis and surgical resection of NPC are difficult because of its deep-seated location and high metastatic tendency. The common therapeutic strategy for patients with advanced NPC employs a combination of ionizing radiation and chemotherapy (¹, ²). Although the modality of cisplatin combined with fractionated radiotherapy has increased the survival rate of patients with NPC, the initial efficacy of the
combined chemo-radiotherapy is often weak because of irradiation-induced multidrug resistance in tumor cells (3, 6).

The mechanisms by which tumor cells develop multidrug resistance after radiotherapy are not fully understood. A number of studies indicated that multidrug resistance gene 1 (MDR1) and its protein product, P-glycoprotein (P-gp), is likely to be the major player mediating drug resistance related to radiotherapy (5, 6). Increased MDR1 mRNA and P-gp protein in CNE1 human NPC cell line correlated with cisplatin resistance after fractionated ionizing irradiation (7). Despite the prominent linkage between multidrug resistances associated with radiotherapy and increased MDR1 level, the mechanisms underlying the regulation of MDR1 expression by ionizing remains unclear. An increased MDR1 level was detected in irradiated CNE1 cells, which correlated with the upregulation of platelet/endothelial cell adhesion molecule 1 (PECAM-1), using polymerase chain reaction (PCR) array (7). Therefore, it was postulated that PECAM-1 might be involved in controlling radiotherapy-related drug resistance.

PECAM-1, also known as CD31, is a 130-kDa glycoprotein belonging to the immunoglobulin superfamily. PECAM-1 is found mainly on platelets, vascular endothelial cells, and several types of leukocytes such as monocytes, neutrophils, and T cells. It mainly serves as an adhesion molecule that facilitates leukocyte transmigration, angiogenesis, and integrin activation (8-10). Studies examining PECAM-1 expression in tumor cells also demonstrated a high frequency of PECAM-1 on a variety of malignant cells derived from hematopoietic cells and malignant vascular tumors (11-13). PECAM-1 expression has also been detected in cells from several types of solid tumors, including breast cancer, colon carcinoma, cervical carcinoma, prostate adenocarcinoma, head and neck squamous carcinoma, and melanoma (14-16). Clinical studies also indicated a close relationship between PECAM-1 expression and cancers.

A recent study identified increased PECAM-1 expression as a prognostic biomarker in the early stage of laryngeal squamous cell carcinoma (17). Another report revealed that the upregulation of genes related to angiogenesis, including PECAM-1, had relevance to brain metastasis in patients with lung cancer and melanoma (18). Moreover, accumulating evidence demonstrated that PECAM-1 functioned not only as a regulator of inflammatory response but also as a major coordinator of multiple signaling pathways controlling cell survival and apoptosis. The emanated signals following PECAM-1 ligation have been implicated in the activation of Ras/Raf/MEK/ERK pathway and PI3K /Akt) signaling pathways, leading to increased expression of Bcl-2 and Bcl-X and decreased expression of Bax (19-21). All these studies suggested an important role of PECAM-1 in controlling cellular apoptosis. However, the impact of PECAM-1 on the acquisition of multidrug resistance of tumor cells treated with ionizing radiation has not been explored.

The aims of the present study were to confirm the role of PECAM-1 in increasing MDR1 expression in CNE1 cell line with multidrug resistance induced by irradiation, and to test the potential effect of targeting PECAM-1 on the cisplatin sensitivity of CNE1 cells subjected to ionizing radiation in both in-vitro and in-vivo settings.

MATERIALS AND METHODS

Cell culture and treatment

The human NPC cell line CNE1 was obtained from the Chinese Academic Cell Bank (Beijing, China) and cultured at 37°C with 5% CO2 using RPMI1640 medium supplemented with 10% fetal bovine serum and Pen/Strep antibiotics. The CNE1 cells were irradiated every other day with a Varian 2300 C/D linear accelerator (Varian, Darmstadt, Germany) using 6-MV X-rays, 2 Gy each time, until the cumulative dose was up to 50 Gy. After irradiation, the cells were harvested weekly to examine the levels of MDR1 mRNA for seven consecutive weeks, and the cells with maximum MDR1 transcript were further screened with limiting dilution assay. A CNE1 subline with both phenotypes of a high level of
MDR1 expression and cisplatin resistance was cloned and designated as CNE1/R as described previously (7).

**RNA isolation and reverse transcription–PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen, NY, USA) based on the manufacturer’s protocol, followed by cDNA synthesis with Moloney Murine Leukemia Virus Reverse Transcriptase (Promega, WI, USA). All primers used for PCR in this study were synthesized by Takara Biotechnology (Dalian, China). The sequences of the primers were as follows: PECAM-1: 5′-ACCAAGATA GCCTCAAGTC-3′ (forward), 5′-TTCACCTCAGAACCCTC-3′ (reverse); MDR-1: 5′-GGTGTGTTGGTGTGTTACA-3′ (forward), 5′-TGGCCAAAATCACAGGTG-3′ (reverse); and β-actin: 5′-CTGACATT AAGGAGAAGCTG-3′ (forward), 5′-CTAGAAGC ATTTGCGGTGGAC-3′ (reverse). PCR products were resolved by 1% agarose gel electrophoresis and photographed with a gel imaging system (Syngene GBOX, UK). The semi-quantitative analysis was based on the densitometry measurement of target bands with NIH ImageJ software.

Real-time quantitative reverse transcription (RT)-PCR (qRT-PCR) was performed using the same primers as earlier with SYBR Premix Taq reagent (Takara Biotechnology, Dalian, China) in iQ5 quantitative PCR machine (Bio-Rad, CA, USA). The relative levels of target gene expression were normalized to β-actin and calculated based on the 2-ΔΔCT method.

**Protein extraction and Western blot analysis**

Whole-cell proteins were extracted with radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktails (Roche, Mannheim, Germany). Fifty micrograms of proteins from each sample were separated by SDS-PAGE electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 5% nonfatty milk and was probed with primary antibodies against human PECAM-1 or P-gp (Abcam, MA, USA) overnight at 4°C and then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. The signals of the target protein were revealed using enhanced chemiluminescence reagent (TransGen Biotech, Beijing, China) and visualized with a luminescence imaging system (Syngene GeneGnome XRQ, UK). Densitometry analysis of each specific protein band was performed using NIH ImageJ software, and the protein levels were expressed as the ratio of normalized densities of target proteins in tested groups to those in the relevant control group.

**Construction of plasmid carrying shRNA**

PECAM-1-specific small hairpin siRNA (shRNA) was synthesized based on the pre-selected sequence of siRNA targeting PECAM-1 and subcloned into a pSilencer2.1-neo shRNA expression vector at BamHI and HindIII sites. The pSilencer2.1 plasmid was provided by Tianjin SaierBio (Tianjin, China), and all other reagents to construct the recombinant plasmids were purchased from Takara Biotechnology (Dalian), including restriction enzymes, DNA, and relevant kits. The whole sequence of shRNA targeting human PECAM-1 was as follows: 5′-GATCCGGACATCTCTGAA CGGAATTCAAGA GTATCTGCG-3′ (sense); 5′-AGCTTTTCCAAAA TTCAGA TACTCTAGAAGCTTCCGAGTTGCCTGGAAC-3′ (antisense). Successful ligation and correct sequence were confirmed by enzymatic digestion analysis and DNA sequencing, respectively.

**Screening clones of CNE1 cells stably expressing PECAM-1 shRNA**

CNE1/R cells were transfected with either a pSilencer empty vector or pSilencer-PECAM-1 shRNA plasmid using Lipofectamine 2000 reagent (Invitrogen, NY, USA). The transfected cells were selected with G418 for 2 weeks, and the survival cells were further screened by the limiting dilution method in the presence of G418. The cell clone of CNE1/R stably expressing PECAM-1 shRNA was designated as pSilencer-siPECAM-1-CNE1/R, while the cell clone with pSilencer empty vector was designated as pSilencer-CNE1/R.
Cell proliferation and apoptosis

Both CNE1/R cells transfected with either PECAM-1 shRNA or control vector were incubated with a medium containing different doses of cisplatin (0.06–1.5 µg/mL) for 24 h and then subjected to cell viability assays. Cell proliferation was measured using the MTT (Sigma, USA) assay. The apoptotic cell death was assessed by staining the cells with an Annexin V–FITC/PI cell apoptosis detection kit (Invitrogen) followed by flow cytometry analysis (FACS101, BD Bioscience, CA, USA) following the manufacturer’s protocols.

Animal studies

For in-vivo studies, 6- to 8-week-old BALB/c athymic nude mice were purchased from the Animal Center of Dalian Medical University and kept in the feeding room of the university’s specific-pathogen-free (SPF) facility. All procedures of animal work were approved by the Institutional Animal Care and Use Committee of Dalian University (registration number: 201308011, Aug. 15, 2013). Twenty-four mice were randomly divided into three groups and inoculated subcutaneously with CNE1/R cells stably transfected with different plasmids (pSilencer-PECAM-1 shRNA or pSilencer empty vector) or no plasmid. Each mouse was injected with 2 × 10⁶ cells in 100 μL of PBS, and tumor formation was monitored. Then, the mice in each group were further divided into two subgroups administered with either cisplatin (6 mg per 10 g body weight) or PBS intraperitoneally at a 4-day interval for 4 weeks. After completing the treatment, all mice were euthanized, and the entire tumor tissues were excised, weighed, and processed for histological examination and protein extraction.

Immunohistochemistry staining

Paraffin-embedded tumor tissues were sectioned at 4-µM thickness. For PECAM-1 and P-gp immunostaining, the tissue sections were first rehydrated and incubated with 3% H₂O₂ solution to block endogenous peroxidase activity and then subjected to antigen retrieval by boiling the slides in 10 mM citrate buffer, followed by blocking with 5% BSA for 1 h at room temperature. The slides were incubated with appropriately diluted rabbit anti-human PECAM-1 or P-gp antibodies (Abcam) at 4°C overnight and then washed and incubated with HRP-conjugated secondary antibody for 30 min at room temperature. The positive reaction was revealed by incubation with 3,3’-Diaminobenzidine (DAB) substrate, followed by counterstaining with hematoxylin.

Statistical analysis

The quantitative data were expressed as mean ± SD. The data were analyzed by one-way ANOVA, and Bonferroni correction was used for multiple paired comparisons with SPSS17 software. A P value <0.05 indicated a statistically significant difference.

RESULTS

Increased MDR1 level in irradiated CNE1 cells correlated with PECAM-1 upregulation

Fractionated ionizing irradiation induced a concomitant upregulation of MDR1 and PECAM-1 mRNA in CNE1 cells. The present study sought to confirm the correlation between PECAM-1 and MDR1 at both transcription and protein levels. Total RNA and whole-cell proteins were extracted from CNE1/R cells derived from a CNE1 clone, stably expressing an increased MDR1 transcript after receiving irradiation. The RT-PCR assay showed that the mRNA level of MDR1 and PECAM-1 was, respectively, three- and twofold higher in CNE1/R cells compared with nonirradiated parental CNE1 cells (figure 1A). Accordingly, the protein expression of P-gp and PECAM-1 increased up to four- and threefold, respectively, in CNE1/R compared with CNE1 cells (figure 1B). These results confirmed and extended a previous observation of concurrent upregulation between MDR1 and PECAM-1 in CNE1 tumor cells receiving ionizing radiation.

Silencing PECAM-1 inhibited the expression of MDR1 mRNA and P-gp protein

A recombinant pSilencer plasmid was constructed to deliver PECAM-1 shRNA and cell
clones stably transfected with either empty vector or plasmid carrying PECAM-1 shRNA to investigate any cause-and-effect connection between PECAM-1 and MDR1 expression. Using quantitative RT-PCR, an 80% downregulation of PECAM-1 mRNA was detected in cells transfected with targeting shRNA compared with control cells with an empty vector (figure 2A). Knocking down PECAM-1 also resulted in a 60% reduction in MDR1 expression compared with vector control cells (figure 2B). In accordance with the mRNA levels, the Western blot assay demonstrated that PECAM-1 shRNA resulted in nearly an 80% reduction in the PECAM-1 level compared with vector control cells (figure 2C), which was accompanied by a similar amount of reduction in the P-gp protein level compared with control cells (figure 2D). Together, these results suggested a role of PECAM-1 in controlling MDR1 and P-gp expression in tumor cells after ionizing irradiation.

Knocking down PECAM-1 increased the sensitivity of CNE1/R cells to cisplatin in vitro

Considering the close relationship between PECAM-1 and MDR1, the study next determined whether knocking down PECAM-1 could sensitize CNE1/R cells to cisplatin treatment. The IC_{50} dosage of cisplatin in CNE1/R cells with or without PECAM-1 shRNA was determined. The MTT assay showed that expression of PECAM-1 shRNA increased the sensitivity of CNE1/R cells to cisplatin-mediated growth inhibition, which was evidenced by the reduction in IC_{50} from 0.643±0.037 μg/mL in cells with control plasmid down to 0.296±0.008 μg/mL in cells with PECAM-1 shRNA (P<0.05). Furthermore, the frequency of apoptotic cells after silencing PECAM-1 also increased compared with that in control cells (figure 3A and 3B). Collectively, these data indicated a major role of PECAM-1 in determining the anti-tumor effect of cisplatin on tumor cells.
subjected to ionizing radiation.

![Figure 3. Silencing PECAM-1 sensitized CNE1/R cells to cisplatin-mediated apoptosis. CNE1/R cells stably transfected with a PECAM-1 shRNA plasmid or an empty vector were treated with indicated doses of cisplatin for 48 h. (A) A representative profile of Annexin V–FITC/PI double staining and FACS analysis. (B) Summarized frequencies of apoptotic cells treated with different doses of cisplatin based on three independent experiments. *Indicates P <0.05, compared with vector control cells.](image)

**Targeting PECAM-1 enhanced cisplatin-induced tumor regression in xenograft mouse models**

Xenograft mouse models were created by subcutaneously inoculating CNE1/R cells stably transfected with PECAM-1 shRNA plasmid, empty plasmid (vector control), or transfection reagent only (nontransfection control) to further validate the effect of targeting PECAM-1 on CNE1/R cells in an *in vivo* setting. After tumor formation, the mice in each group were treated with either cisplatin or PBS for 4 weeks. As shown in figure 4A and 4B, tumors derived from CNE1/R cells carrying PECAM-1 shRNA exhibited smaller tumor size and reduced tumor weight (0.68±0.03 g) compared with those found in the other two groups, implying that targeting PECAM-1 alone retarded tumor growth.

Cisplatin treatment inhibited tumor growth in mice from all three groups; the most prominent tumor regression by cisplatin treatment was found in tumors derived from CNE1/R cells carrying PECAM-1 shRNA. As shown in figure 4B, the average tumor weight in the group with PECAM-1 shRNA was 0.25±0.04 g in the presence of cisplatin, which was much less than that found in either the vector control group (0.99±0.17 g) or the nontransfected group (1.04±0.09 g).

**Figure 4. Silencing PECAM-1 inhibited tumor growth and enhanced the efficacy of cisplatin treatment in xenograft mouse models. Xenograft mouse models were established by subcutaneously inoculating CNE1/R cells with no transfection or stably transfected with an empty vector or PECAM-1 shRNA plasmids. (A) Image shows the gross appearance of excised tumors from each indicated group of animals treated with either cisplatin or saline. (B) Comparison of average tumor weight among indicated groups. *Indicates P <0.05, **P <0.01, compared with the two indicated control groups.**

**Tumor regression correlated with the diminished level of PECAM-1 and P-gp proteins**

Immunohistochemical staining was performed using tumor tissues from all three groups described earlier to confirm the relevance of tumor regression to diminished PECAM-1 and P-gp levels. As shown in Figure 5A, tumors from both no-transfection and vector control groups exhibited the positive staining of PECAM-1 and P-gp proteins, revealed by dark
brown labeling in the cytoplasm and on the cell membrane of tumor cells. In contrast, tumors with PECAM-1 shRNA displayed much weaker staining for both PECAM-1 and P-gp proteins. The protein levels of PECAM-1 and P-gp were also examined by Western blot analysis with proteins extracted from tumor tissues. Densitometry analysis of the blots showed that tumors with PECAM-1 shRNA expressed nearly 80% reduction in the levels of both PECAM-1 and P-gp compared with that in tumors from cells with either control plasmid or no transfection (figure 5B), thus further supporting the role of PECAM-1 in controlling P-gp expression and indicating that tumor regression had prominent relevance to PECAM-1 inhibition.

**DISCUSSION**

The present study extended previous observations by showing that an increased PECAM-1 level was involved in controlling MDR1 and P-gp expression, thus playing a critical role in the acquisition of drug resistance by CNE1 cells subjected to ionizing radiation. This study also demonstrated that silencing PECAM-1 expression increased the sensitivity of irradiated CNE1 cells to cisplatin-mediated cell death and tumor regression.

The frequency of PECAM-1 expression on solid tumor cells was relatively low compared with cells from hematopoietic lineages. Using RT-PCR and Western blot analysis, low but detectable levels of both PECAM-1 mRNA and protein were observed in the nonirradiated CNE1 cell line. This phenomenon was not a surprise because cell lines from epithelial tissues, including head and neck squamous carcinoma, cervical carcinoma, and breast carcinoma, have been reported to express PECAM-1 mRNA and protein (15). The expression of both PECAM-1 transcript and protein was significantly upregulated by fractionated ionizing irradiation, indicating that an increased PECAM-1 level might serve as a new biomarker of tumor cells undergoing stress induced by ionizing irradiation.

Increased expression of MDR1 and P-gp is one of the most prominent phenotypes shared by many tumor cells with intrinsic or acquired drug resistance (22, 23). The fact that ionizing radiation enhances MDR1 and P-gp expression has also been documented by several reports including the present study (5, 7). The data from this study confirmed the correlation between the expression levels of PECAM-1 and MDR1, indicating cooperation between these molecules in determining the drug resistance of tumor cells following exposure to ionizing radiation. Silencing PECAM-1 by specific shRNA in irradiated CNE1 cells also abridged the expression of MDR1 transcript and P-gp protein, highlighting that PECAM-1 might serve as an upstream regulator of MDR1. This is the first evidence implicating the role of PECAM-1 in controlling MDR1 expression in tumor cells after radiotherapy.

The detailed signaling pathways by which PECAM-1 affected MDR1 expression were not elucidated in the present study. However, it was considered that PECAM-1 regulated MDR1 most
likely in an indirect way, given the fact that PECAM-1 itself is not a transcription factor, and that PECAM-1 ligation can engage multiple signaling pathways. Several transcription factors and kinases, such as p53, NF-kB, and PI3K/Akt signaling axis, have been reported to control MDR1 expression \[^{24-26}\]. The signals emanated from PECAM-1 ligation can directly or indirectly activate NF-kB and Akt signaling pathway \[^{9}\]. As a result, these events may contribute to the enhancement of MDR1 transcription and P-gp protein synthesis. The treatment of irradiated CNE1 cells with LY294002, a potent PI3K inhibitor, resulted in a concurrent reduction in the levels of phosphorylated Akt and P-gp protein (unpublished data). Nevertheless, other complex interactions between PECAM-1 and MDR1 may also exist, which need further exploration.

The evidence from this study supported the critical role of PECAM-1 in tumor cell survival. PECAM-1 shRNA treatment inhibited CNE1 cell proliferation and increased the sensitivity of irradiated CNE1 cells to cisplatin \textit{in-vitro}. This evidence was further corroborated by the \textit{in-vivo} study using xenograft mouse models by inoculating CNE1/R cells carrying PECAM-1 shRNA followed by cisplatin treatment. Collectively, these results suggested that PECAM-1 might be an attractive target for cancer therapy and an important regulator in determining drug resistance of tumor cells after ionizing irradiation. This notion was also supported by recent studies showing that the growth of mantle cell lymphoma in a xenograft model was associated with upregulated PECAM-1 expression \[^{28}\]. This study was supported in part by a grant from the National Natural and Scientific Foundation of China, No. 30970869. We thanks Drs Qing-ping Liu and Jian-zhong Qin for valuable suggestion to this study. The authors would like to thank MJEeditor (www.mjeditor.com) for its linguistic assistance during the preparation of this manuscript.

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