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LETTER TO THE EDITOR

5-FU pretreatment potentiates cisplatin-induced apoptosis through up-regulation of thrombospondin-1 in head and neck squamous cell carcinomas

Bei Xu · Peng Liu

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We read with interest the recent article in *Cancer Chemother Pharmacol* on treatment schedules with 5-FU and cisplatin in head and neck squamous cell carcinomas (HNSCCs) by Kei Ijichi et al. [1]. The authors concluded that long arrest in S-phase might be one of the mechanisms by which prior 5-FU treatment followed by cisplatin demonstrates a higher cytotoxicity than the reverse sequential treatment. Here, we report that 5-FU pretreatment could potentiate cisplatin-induced apoptosis through up-regulation of a secreted component of extracellular matrix, thrombospondin-1 (TSP-1), which provide a novel explanation for the advantage of 5-FU pretreatment in the combination therapy in HNSCCs.

We first checked the effect of 5-FU treatment on TSP-1 expression in two commonly used HNSCC cell lines, SCC-9 and SCC-25. Both cell lines were incubated in 5-FU at concentrations from 0 to 2 μM for 72 h and TSP-1 was measured at mRNA and protein level. Previous studies [2] showed that upon its induction by various stimuli, TSP-1 is mostly secreted into the extracellular culture medium. Thus, we detected its protein production using enzyme-linked immunosorbent assay (ELISA). As shown in Fig. 1a, b, 5-FU markedly up-regulated both TSP-1 transcription and protein production in the two cell lines tested. To evaluate the potential role of TSP-1 up-regulation in the subsequent cisplatin-induced apoptosis, we used shRNA technology to induce TSP-1 gene silencing and established stable transfectants in these two cell lines. TSP-1 gene knockdown with shRNA and its effect on TSP-1 protein production were confirmed in Fig. 1c, d. We then treated SCC-25 stable transfectants expressing TSP-1 shRNA with two schedules: 72 h 5-FU (2 μM) treatment followed by 24 h cisplatin (10 μM) and the reverse sequential treatment. As shown in Fig. 1e, the generation of apoptosis by subsequent cisplatin treatment was significantly decreased by the transfection of shRNA targeting TSP-1, suggesting that the synergistic effect of 5-FU pretreatment was at least partially mediated by TSP-1. However, TSP-1 gene silencing had no effect on apoptosis induced by the reverse schedule. It is interesting to note that 24 h pretreatment with 10 μM cisplatin did not change TSP-1 expression at mRNA or protein level ($P > 0.05$, Fig. 1f, g).

TSP-1 is a multimodular, 420 kDa homotrimeric matricellular glycoprotein that participates in cellular response to growth factors, cytokines and injury. It regulates cell proliferation, migration and apoptosis in a variety of physiological and pathological settings, including wound healing, inflammation, angiogenesis and neoplasia [3–5]. TSP-1 has been described to induce a caspase-dependent cell death mediated by CD36 in leukemic HL-60 cell line [6]. It is also able to trigger a caspase-independent cell death in B-chronic lymphocytic leukemia and promyelocytic leukemia through binding to CD47 [2, 7, 8]. TSP-1 also induces CD47-mediated killing of breast cancer cells acting via heterotrimeric Gi-dependent inhibition of protein kinase A activity [9]. For HNSCCs, TSP-1 has been identified to be downregulated in HNSCC patient specimens using tissue microarrays technology [10]. However, little is known about the role of TSP-1 in the control of apoptosis in HNSCCs.

B. Xu
Department of Internal Medicine,
The First Affiliated Hospital of Nanjing Medical University,
Nanjing, People's Republic of China

P. Liu (✉)
Department of Hematology,
The First Affiliated Hospital of Nanjing Medical University,
300 Guangzhou Rd, 210029 Nanjing, People's Republic of China
e-mail: liupeng8888@yahoo.com.cn

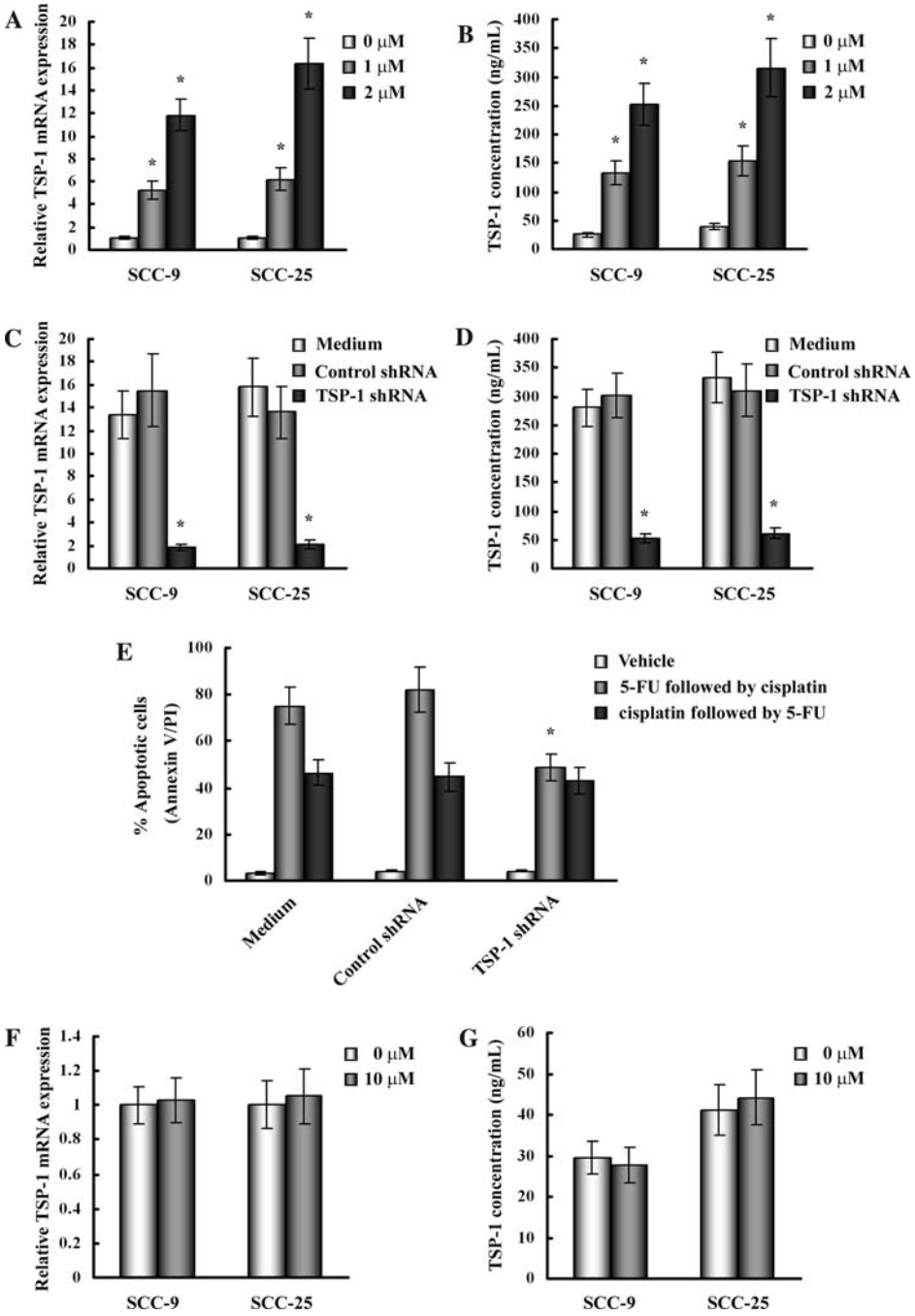

Fig. 1 **a** SCC-9 and SCC-25 HNSCC cell lines were incubated in 5-FU at indicated concentrations for 72 h, total RNA was isolated and TSP-1 mRNA expression relative to 18S rRNA was determined by quantitative real time RT-PCR. The following primers were used for TSP-1 mRNA amplification: forward 5′- AACATGCCACGGCCAACAAA-3′ and reverse 5′-TGCACTTGGCGTTCTTGTTGC-3′. **b** TSP-1 protein secreted in the cultured supernatant was measured by ELISA (Chemicon International, Temecula, CA) after the same treatment as Fig. 1a. c, d, SCC-9 and SCC-25 stable transfectant cells expressing control shRNA or shRNA against TSP-1 were exposed to 2 μM 5-FU for 72 h, after which relative TSP-1 mRNA expression and TSP-1 protein secretion were determined by quantitative real time RT-PCR (**c**) and ELISA (**d**), respectively. The sequence of shRNA targeting TSP-1: CCGGCTCTCAAGAAATGGTGTTCTTCTCGAGAAGAACACCATTTCTTGAGAGTTTTTG. The sequence of control shRNA: CCGGCAACAAGATGAAGAGCACCAACTCGAGTTGGTGCTCTTCATCTTGTTGTTTTT. **e** SCC-25 stable transfectants expressing control shRNA or TSP-1 shRNA were treated with two schedules: 72 h 5-FU (2 μM) treatment followed by 24 h cisplatin (10 μM) and the reverse sequential treatment. Additional 24 h later, apoptotic cells were monitored by annexin V/PI staining (BD Pharmingen) and flow cytometry. **f** SCC-9 and SCC-25 HNSCC cell lines were treated with 10 μM cisplatin for 24 h, total RNA was isolated and TSP-1 mRNA expression relative to 18S rRNA was determined by quantitative real time RT-PCR. **g** TSP-1 protein secreted in the cultured supernatant was measured by ELISA after the same treatment as Fig. 1f. Results are shown as mean ± SD of triplicates samples. * $P < 0.01$

5-FU is a commonly used antitumor drug in chemotherapy against various solid tumors. Recent studies showed that 5-FU treatment augments TSP-1 promoter activity, with the subsequent production of TSP-1 mRNA and protein in human colon carcinoma cells and breast cancer cells [11, 12]. Our data presented here indicate for the first time that 5-FU pretreatment significantly enhances cisplatin-induced apoptosis through TSP-1 up-regulation in HNSCCs, which provides the molecular basis for the more effective treatment sequence of 5-FU and cisplatin-based chemotherapy.

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