

Contribution of Nitric Oxide and Epidermal Growth Factor Receptor in Anti-Metastatic Potential of Paclitaxel in Human Liver Cancer Cell (HebG2)

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ABSTRACT

Background: Paclitaxel is a general antineoplastic drug used against different types of experimental and human tumors. Several anti-cancer drugs have been shown to stimulate nitric oxide (NO) production, which has been shown to affect many aspects of tumor biology.

Objective: This study was initiated to determine if paclitaxel stimulates NO production in HebG2 cells, and if so, whether NO interferes with the metastatic potential of HebG2 cells and contributes to paclitaxel cytotoxicity. In addition, we sought to determine the relationship between NO production and the expression of epidermal growth factor receptor (EGFR) and matrix metalloproteinases (MMPs) in HebG2 cells.

Materials and Methods: The effects of paclitaxel (0.1-1000nM) on surviving fraction, NO production and the expression of EGFR, MMP-2 and MMP-9 were studied in human liver cancer cells (HebG2).

Results: Paclitaxel resulted in a significant dose-dependent decrease in the surviving fraction of HebG2 cells. A 62% and 86% decrease in the surviving fraction was attained at 10nM and 100nM paclitaxel, respectively. Paclitaxel produced a significant increase in NO production, starting from 1nM. A 64% and 111% increase in NO production was attained after exposure to 10nM and 100nM of paclitaxel, respectively. In all of the HebG2 cells treated with paclitaxel (1.0-1000nM) mRNA specific for EGFR, MMP-2 and MMP-9 were undetectable. However, untreated HebG2 cells and those treated with paclitaxel (0.1nM) expressed mRNA specific for these markers.

Conclusion: This study suggests that: (1) increased production of NO may contribute to paclitaxel's cytotoxicity against HebG2 cells, (2) paclitaxel may inhibit tumor metastasis via inhibition of the expression of EGFR and MMPs and (3) an inverse correlation exists between NO production and expression of EGFR and MMPs.

Key Words: Epidermal growth factor - Paclitaxel - Liver - Cancer cells.

INTRODUCTION

In the last decade, the use of taxoid anti-cancer drugs against metastatic liver, breast and ovarian cancer, as well as non-small lung cancer, melanoma and other cancers, has been gaining acceptance. Paclitaxel was the first among taxoids to be isolated from the bark of the Western Pacific yew, *taxus brevifolia* [1,2]. Paclitaxel is a unique cytotoxic antineoplastic drug that results in tumor cell kill by producing excessive polymerization of tubulin and dysfunction of microtubules [3-5]. Several antineoplastic agents, including cisplatin, hydroxyurea, doxorubicin and relaxin, have been shown to stimulate nitric oxide (NO) production [6-8]. It has been demonstrated that tumor cells are susceptible to NO cytostasis [9]. Nitric oxide may influence several aspects of tumor biology, including modulation of cell growth, apoptosis, differentiation, angiogenesis and metastatic capability [9]. NO performs this function through several mechanisms, including an inhibition of DNA synthesis [10], mitochondrial respiration [11], cytochrome p-450 activity [12] and an interference with iron-sulfur proteins [13].

Metalloproteinases (MMPs) enhance tumor cell invasion by degrading extra-cellular matrix proteins, by activating signal transduction cascades that promote motility [14] and by solubilizing extra-cellular matrix-bound growth factors [15,16]. MMPs also regulate tumor angiogenesis and may be required for the angiogenic switch that occurs during neovascularization [17]. Also, the levels of MMP-2 and MMP-9 are highly correlated with the histological grade of malignancy [18]. The EGFR is a MW: 170.000 kdal

transmembrane phosphoglycoprotein whose over-expression has been shown to correlate with decreased disease-free survival and increased metastasis in many tumors [19-21]. EGFR has at least seven cognate ligands, including EGF itself [22]. Aberrant expression of EGFR and one or more of its ligands may result in autocrine or paracrine activation, leading to uncontrolled cell growth, transformation and tumor progression [23]. Increased invasion and metastatic potentials have been associated with the presence of EGFR in a number of different tumor cells [24]. It is apparent that MMPs expression in carcinomas involves complex interaction between extra-cellular matrix (ECM), cytokines and cell surface receptors. Among these, EGFR activation has been shown to correlate with some members of the MMP family [25]. Although the interaction of paclitaxel with tubulin has been well characterized, the exact mechanism whereby paclitaxel induces its cytotoxicity and antimetastatic effect is not well understood. Therefore, this study was initiated with the following specific aims: (1) to determine whether paclitaxel stimulates NO production in HebG2 cells, and if so, whether NO interferes with the metastatic potential of HebG2 cells and contribute to paclitaxel's cytotoxicity and (2) to determine the relationship between NO production and the expression of EGFR and MMPs in HebG2 cells.

MATERIAL AND METHODS

Materials: Paclitaxel (taxel 30mg vials, Bristol-Meyer) was a generous gift from the National Cancer Institute drug store. The HebG2 human liver cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). RPMI medium, Fetal Calf Serum (FCS), antibiotics for cell culture, trypsin solution and tissue culture plasticware were purchased from Costar (Milan, Italy). All other chemicals were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Evaluation of Cellular Cytotoxicity: The cytotoxic activity of paclitaxel against HebG2 cells was determined using Sulphorhodamine-B assay according to Skehan et al. [26]. In brief, HebG2 cells were seeded into 96-well microtiter plates at a concentration of 5×10^4 cells/well in fresh medium and left to attach to the plate for 24 hours. Cells were then incubated for 48 hours in the absence (control) and in the pres-

ence of paclitaxel at the noted concentrations (0.1-1000nM). Following 48-hour exposure to paclitaxel, cells were fixed with 50% cold TCA for one hour, stained for 30 minutes with 0.4% Sulphorhodamine-B and then washed with 1% acetic acid. The plates were then air-dried and the optical density of each well was measured spectrophotometrically at 564nm using the ELISA microplate reader (Meter tech. Σ 960, USA).

Evaluation of Nitric Oxide Production: Nitric oxide production was performed by determining the amount of nitrite, a stable end product of NO metabolism in HebG2 cell homogenates according to Bani et al. [6]. The cells were seeded into 6-well plates at 10^6 concentration, allowed to grow for 24 hours, and then incubated for 48 hours in the absence (control) and in the presence of paclitaxel at the noted concentrations (0.1-1000nM). To avoid interference by L-arginine contained in FCS on NO production by the cells, the experiments were carried out with steady-state medium. At the end of the treatments, the cells were detached from culture plates, centrifuged, resuspended in 500ul of PBS, and sonicated. The amount of nitrite was measured spectrophotometrically by the Griess reagent. Briefly, the samples were added to nitrate reductase (276 miliunits) and NADP⁺ (40uM) and then allowed to react with Griess reagent (aqueous solution of 1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 2.5% H₃PO₄) to form a stable chromophore. Absorbance was measured at 546nm. The values were obtained by comparison with standard concentrations of sodium nitrite and expressed as the amount of nitrite per mg of protein.

RNA Extraction and cDNA: Total cellular RNA was extracted from the HebG2 cell line by using RNA clean (AGS GmbH, Heidelberg, Germany), according to the manufacturer's instructions, and dissolved in Diethyl Pyro Carbonate (DEPC) water and stored at -70°C until assaying. First-strand cDNA was synthesized using M-colony murine leukemia virus reverse transcriptase (Life Technologies, Inc). RNA (1ug) was added to 200 units of enzyme, 2ul 10 x reaction buffer (500mM tris HCl, pH 8.3, 70mM KCl, 100mM DTT, 30mM MgCl₂), 1mM deoxyribonucleoside triphosphates (Amersham Pharmacia Biotech, Piscataway, NJ,

USA), 20 units, RNA-sin (Promega Cooperation, Madison, WI, USA), 5uM random hexamers (Roche Diagnostics, Mounheim, Germany), and 1uM antisense primer to a final volume of 20ul. The cDNA synthesis was performed at 37°C for 60 minutes. After heat inactivation at 95°C for 10 minutes, 2ul cDNA were subjected to PCR analysis.

Polymerase Chain Reaction and Gel Electrophoresis: Specific cDNA sequences were amplified in a reaction mixture composed on 2ul cDNA, 5ul 10 x PCR buffer (100mM tris HCl, pH 8.3, 500mM KCl, 15mM MgCl, 0.1% gelatin), 50uM dNTPs, 400nM of each specific sense and antisense primers and 1.5 units AmpliTaq DNA polymerase (Perkin Elmer, Warrington, UK) in a volume of 50ul. The second round of PCR using nested primers and 1ul of outer product was performed identically, except that dNTP concentration was increased to 800nM. All primers were synthesized at the Pharmacia, St. Albans, UK. The primer sequences were listed in Table (1). The cycling condition for EGFR PCR were performed for 30 cycles

consisting of 5 cycles of 30 seconds at 94°C, 45 seconds at 60°C and 45 seconds at 72°C, and 25 cycles of 30 seconds at 94°C, 45 seconds at 55°C and 45 seconds at 72°C in a GeneAmp PCR system 9700 Perkin-Elmer. The samples were heated for 10 minutes at 94°C before the first cycle. The PCR conditions for the nested PCR reaction were similar to those for the first round PCR with the following exceptions: (1) Primers A and C were used for the amplification and (2) the total number of PCR cycles were 35 (5+30 cycles). For MMP-2, 30 cycles (one minute at 94°C, one minute at annealing temperature 58°C and two minutes at 72°C for the first cycle, 93 minute denaturation) was performed, and then all samples were incubated for an additional four minutes at 72°C. For MMP-9, PCR included 35 cycles, with each cycle consisting of 30 seconds of denaturation at 94°C, one minute at 68°C and one minute at 72°C. The RT-PCR products were analyzed in 2% agarose gels, stained with ethidium bromide. DNA ladder (Life Technologies, USA) was used as a size marker.

Table (1): The primer sequences used is PCR for the detection of EGFR, MMP-2 and MMP-9.

Primer	Product size	Sequence (5' - 3')
EGFR	Outer 322	(A) 5-TCTCAGCAACATGTCGATGG (B) 5-TCGCACTTCTTACACTTGCG
EGFR	Inner	(C) 5-TCACATCCATCTGGTACGTG
MMP-2	447	5-ACCTGGATGCCGTCGTGGAC 5-TGTGGCAGCACCAGGGCAGC
MMP-9	640	5-GGTCCCCCACTGCTGGCCCTTCTACGGCC 5-GTCCTCAGGGCACTGGAGGATGTCATAGGT

RESULTS

Fig. (1) shows the percent of the surviving fraction of HebG2 cells after 48 hours of exposure to different concentrations of paclitaxel (0.1-1000nM). Sixty two and 86% decrease in surviving fraction were reached at 10nM and 100nM paclitaxel, respectively.

An involvement of NO in the response of HebG2 cells to paclitaxel was evidenced by the determination of nitrite in the cell homogenates. As shown in Fig. (2), paclitaxel resulted in a significant increase in NO production, starting from 1nM. A 64% and 111% increase in NO

production was obtained after exposure to 10nM and 100nM of paclitaxel, respectively.

Fig. (3) shows marker expression in the HebG2 cell line treated with paclitaxel. The untreated HebG2 cells were taken as a positive control for examining the expression of EGFR by RT-PCR (Fig. 3-A), MMP-2 (Fig. 3-B), and MMP-9 (Fig. 3-C). In all of the HebG2 cells treated with paclitaxel (1.0-1000nM), mRNA specific for EGFR, MMP-2 and MMP-9 were undetected. However, HebG2 cells treated with paclitaxel (0.1nM) expressed mRNA specific for the previously mentioned markers.

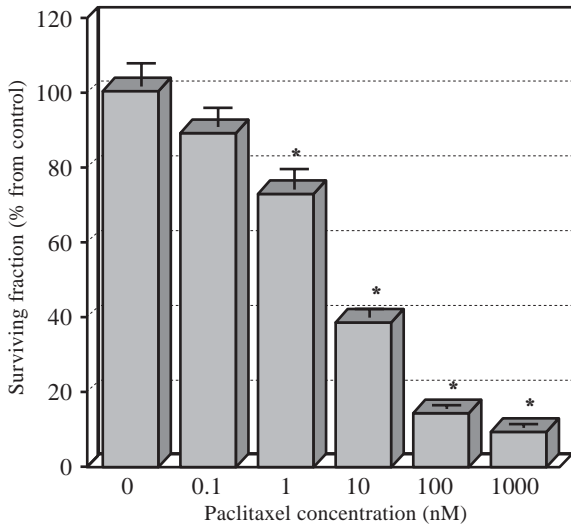


Fig. (1): Effects of different concentrations of paclitaxel (0.1-1000nM) on surviving fraction of HebG2 cells. Data are presented as a mean \pm SE of four separate experiments. * indicates significant difference from control with a *p* value < 0.05.

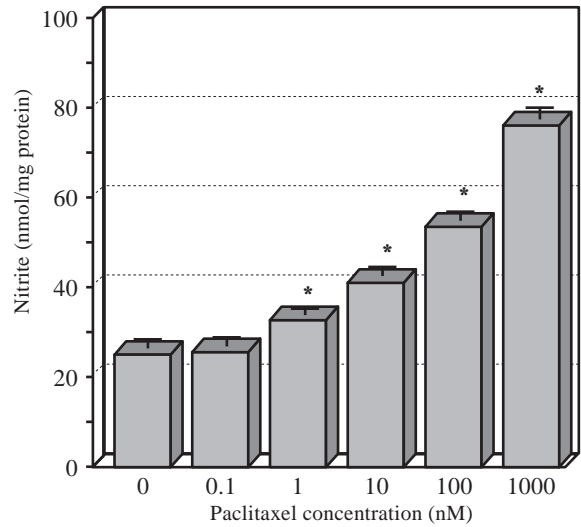


Fig. (2): Effects of different concentrations of paclitaxel (0.1-1000nM) on nitric oxide production in HebG2 cells. Data presented are mean \pm SE of four separate experiments. * *p* value \leq 0.05 are considered significant.

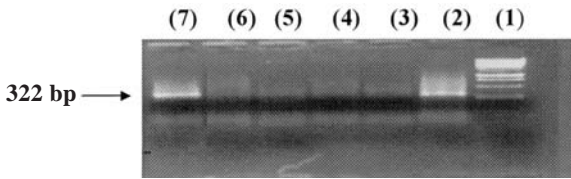


Fig. (3-A): RT-PCR identified 322bp fragment of cDNA coding. Electrophoretic gel: Lane 1 showing 1kb DNA ladder marker, Lane 2 showing EGFR positive result for control untreated HebG2 cells, Lanes 3-6 showing HebG2 cells treated with paclitaxel (1000, 100, 10, 1nM, respectively) and showing negative results, Lane 7 showing HebG2 cells treated with 0,1nM paclitaxel and showing positive results for EGFR.

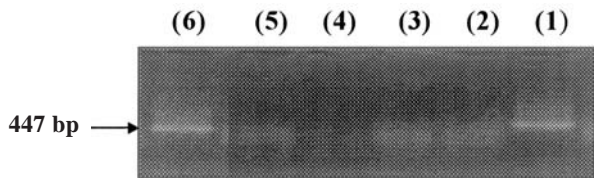


Fig. (3-B): RT-PCR identified 447bp fragment of cDNA coding. Electrophoretic gel: Lane 1 showing MMP-2 positive result for control untreated HebG2 cells, Lanes 2-5 showing HebG2 cells treated with paclitaxel (1000, 100, 10, 1nM, respectively) and showing negative results, Lane 6 showing HebG2 cells treated with 0.1nM paclitaxel and showing positive results for MMP-2.

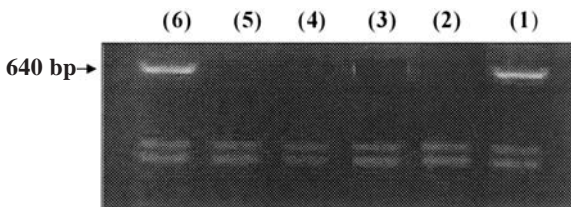


Fig. (3-C): RT-PCR identified 640bp fragment of cDNA coding. Electrophoretic gel: Lane 1 showing MMP-9 positive results for control untreated HebG2 cells, Lanes 2-5 showing HebG2 cells treated with paclitaxel (1000, 100, 10, 1nM, respectively) and showing negative results, Lane 6 showing HebG2 cells treated with 0.1nM paclitaxel and showing positive results for MMP-9.

DISCUSSION

Data from this study demonstrate that paclitaxel stimulated NO production and decreased the surviving fraction of HebG2 cells in a dose-dependent fashion. These results suggest that NO may contribute to paclitaxel's anti-tumor activity. Previous studies have reported that many anti-cancer drugs stimulate NO production and that their cytotoxic activity and organ toxicity are NO-dependent [6,7,8,27]. NO is a multi-functional messenger molecule derived from the amino acid L-arginine in a reaction catalyzed by nitric oxide synthase [28]. NO may affect cell proliferation via non-apoptotic mechanisms,

such as the inactivation of iron/sulfur-containing enzymes responsible for mitochondrial respiration [9] or the inhibition of ribonucleotide reductase [29]. NO also enhances cellular oxidative injury [30], affects drug metabolizing enzymes [31] and inhibits P-glycoprotein function with consequent increase in the accumulation of anti-cancer drugs in the tumor cells [32].

A role for the EGFR signaling pathway in tumor cell invasion and metastasis has been suggested in various tumor cell types [24,33]. Transmigration of ECM is considered to be dependent on three properties: (1) matrix recognition/adhesion, (2) proteolytic ECM remodeling/degradation and (3) active movement through the resulting defect. EGFR signaling has been implicated in modulating cell phenotypes that control all three aspects on invasion. In this study, we focused on proteolytic degradation, especially that mediated by MMPs. This protease family includes collagenases, gelatinases, stromelysins, membrane type MMPs and additional enzymes, such as matrilysin (MMP-7). These enzymes differ in substrate specificity, regulation, tissue-specific expression, and the potential interactions with additional MMP and tissue inhibitor matrix metalloproteinase (TIMP) family members. Differential protease production would be expected to result in different invasion properties [24]. Our results suggest that paclitaxel inhibits the expression of EGFR, and hence, the expression of MMP-2 and MMP-9. This association between EGFR signaling and MMPs activation is consistent with the work of Cox, et al. [34], who showed that a significant proportion of non-small cell lung cancers co-express MMP-9 and EGFR. There are multiple mechanisms that may be involved in EGFR-mediated MMP-2 and MMP-9 activation, including regulation of transcription, localization of pro-MMP-9 to the leading edge of invasive cells and proteolytic cleavage of their proenzymes [35-40]. In squamous cancer cells, EGFR signaling promotes MMP-9 expression, via persistent activation of the ERK pathway [41].

In this study, the observed increase in NO production by paclitaxel was dose-dependent (Fig. 2) and parallel to the decreased expression of EGFR and MMPs (Fig. 3). These results suggest that paclitaxel may inhibit the expression of EGFR through enhancement of NO production with the consequent inhibition of MMPs

expression and metastasis. Our results are in close agreement with the data presented by Dong, et al. [42], who reported that the increased production of NO in K-1735 murine melanoma cells decreased its metastatic potential via NO-induced vasodilatation and inhibition of platelet aggregation with the consequent decrease in the arrest of tumor cell emboli in capillary beds, and hence, a decrease in metastasis. Moreover, NO has been shown to regulate cell proliferation and apoptosis. Our results showed that NO production increased with the increment of paclitaxel concentrations (Fig. 2), Silvia, et al. [43] showed that NO inhibited DNA synthesis by a cGMP-independent mechanism and that the EGFR is s-nitrosylated. The inhibition of EGFR transphosphorylation by NO was a concentration and time-dependent process, and was inversely proportional to the half-life of NO of the donors. NO may also directly affect the secretion of MMP-9 and the MMP-2 activation through enhancement of TIMP-2 secretion [44].

In conclusion, results of the present study suggest that: (1) increased production of NO may contribute to paclitaxel's cytotoxicity against HebG2 cells, (2) paclitaxel may inhibit tumor metastasis via inhibition of the expression of EGFR and MMPs and (3) an inverse correlation exists between NO production and expression of EGFR and MMPs.

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