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Combined effects of radiotherapy and photodynamic therapy on an *in vitro* human prostate model

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Human prostate cancer cells were evaluated for growth after photodynamic therapy, radiotherapy, and combined treatment. Indocyanine green was tested as a photosensitizer and radiosensitizer.

Two human cell lines were used: PC-3 derived from prostate carcinoma, and EPN derived from normal prostate tissue. The light source used for the photoactivation experiments was a diode laser peaked at 805 nm. The light dose incident on cells was 108 J/cm^2. Ionizing radiation was produced by a linear accelerator, and the dose was 2, 4 and 6 Gy. Cytotoxicity was evaluated by measuring the colony forming ability of cells.

Our results show that indocyanine green induces cell death by photoactivation, but it does not act as a radiosensitizer if used with ionizing radiation. The combined treatment of photodynamic therapy and radiotherapy produces an additive effect which does not depend on the sequence of the two treatments.

Combined treatments could be more useful since they allow the reduction of the ionizing radiation dose to obtain the same effect as one obtainable by radiotherapy alone.

Prostate cancer is the most common cancer in men in the Western countries, and it is the second leading cause of death after cardiovascular diseases. Although considerable effort has been devoted to finding a minimally invasive therapy for prostate cancer in recent years, the appearance within the tumor of androgen insensitive cells often annuls the po-

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Abbreviations: ICG, indocyanine green; PDT, photodynamic therapy; RT, radiotherapy.

tential efficacy of hormonal therapy. In fact, prostate cancer begins as an androgen-dependent tumor that undergoes clinical regression in response to pharmacological or surgical strategies that reduce testosterone. Despite these treatments, in most cases the cancer re-grows in an androgen or hormone-independent form (Repetto *et al.*, 1998). In these cases radiotherapy (RT) does not work properly and acute side-effects and late complications are, unfortunately, dose-dependent (Severson *et al.*, 1995; Dearnaley, 2001). Besides, radiotherapeutic treatment can induce radioresistance of tumor cells. In prostate cancer the radioresistance is attributed to a loss of the apoptotic response (Szostak & Kyprianou, 2000). Therefore, apoptosis becomes a limiting factor for the radiotherapeutic effectiveness on prostate cancer.

To increase the apoptotic response, attention has focused on the development of new therapeutic treatments that combine radiotherapy with chemical or thermal agents, and recently, with photodynamic therapy (PDT).

Since PDT and RT induce damage on different targets, a synergism between them in killing cells might produce better results (Ramakrishnan *et al.*, 1990). PDT and RT interaction should enhance the therapeutic effect, thus reducing the ionizing radiation dose and thereby lowering the potential side effects.

Recently, numerous efforts have been made in searching for new and more effective photosensitizing agents (second-generation agents) that have the following characteristics to improve PDT application: low toxicity in the dark, preferential accumulation in tumor tissue, good water-solubility for the administration of aqueous solutions, and high absorption in the therapeutic window (650–850 nm) where maximum light penetration in tissues occurs (Jori, 1996).

Indocyanine green (ICG) has recently attracted great attention because of its low toxicity, rapid excretion and absorbance around 800 nm (Fox & Wood, 1960; Henschen *et al.*, 1993; Fagrell, 1995; Owens, 1996; Brancato & Trabucchi, 1998; Desmettre *et al.*, 1999; El-Desoky *et al.*, 1999; Wietasch *et al.*, 2000). Furthermore, its photodynamic action on cancer cells *in vitro* has been observed recently (Chong *et al.*, 1993; Szeimies *et al.*, 1994; Fickweiler *et al.*, 1997; Bäumler *et al.*, 1999).

In order to evaluate whether PDT could be integrated with radiotherapy of prostate cancer we first tested the photosensitizing properties of ICG on a system of normal and neoplastic human prostate cells, and then associated ICG-based PDT with radiotherapy.

MATERIALS AND METHODS

Chemicals and cell lines. Indocyanine green purchased from Sigma-Aldrich (Milan, Italy). The stock solution was obtained by dissolving 100 μg of indocyanine green powder in 10 ml of sterilized distilled water. All following dilutions were obtained in culture medium.

The PC-3 cell line was obtained from a prostate carcinoma (Kaighn *et al.*, 1979). Epithelial (EPN) cells were a primary culture from normal prostate tissue (Sinisi *et al.*, 2002). The culture medium for PC-3 cell line was DMEM with 10% fetal calf serum (FCS, Flow Laboratories, Glasgow, U.K.) and 2 mM L-glutamine added. The culture medium for EPN cells was HAM-F12 with 3% FCS and 2 mM L-glutamine added. Both cell lines were grown at 37°C in 5% CO_2 atmosphere.

Cell uptake of indocyanine green. Measurement of the amount of indocyanine green (ICG) in the cells was carried out as previously described by Carin and Pardini (1992). Briefly, 15×10^4 cells were incubated for 2 h with ICG (3 μg/ml), trypsinized, and cell number was determined by counting cell suspension in a Neubauer hemocytometer. Cell suspensions were centrifuged (1020 r.p.m. for 5 min), and the pellets washed in phosphate-buffered saline (PBS) prior to ICG ex-

traction in aqueous 90% acetone. The ICG content was determined by absorbance at 779 nm using the molar absorption coefficient of $2.28 \times 10^5 M^{-1} cm^{-1}$.

Clonogenic assay. PC-3 and EPN cells were plated at about 500 cells/well and incubated for 24 h to allow cells to adhere. Then some wells were treated as described in the following sections, and others were used as controls. All the cells were allowed to grow for 10 days, and colonies were stained with 0.5% methylene blue in 70% ethanol. Colonies with more than 50 cells were counted. The surviving fraction was calculated as the ratio of colony numbers in treated samples relative to control samples. The survival data, expressed as percentage, are the mean of three independent experiments.

Irradiation sources. The light source used for the photoactivation experiments was a diode laser (Quanta System, Italy) peaked at 805 nm. The fluence rate incident on cells was measured by a power-meter (Ophir model DGX 10) and was 120 mW/cm^2.

Photons at 6 MeV were produced by a linear accelerator GE 43 SATURNE. Cells were irradiated at a distance of 70 cm from the photon source with a dose rate of 2 Gy/min.

Irradiation procedure. For photoactivation-only experiments cells were incubated for 2 h in a solution of indocyanine green whose concentration ranged from 1–3.5 μg/ml. After incubation the solution was replaced with fresh drug-free culture medium. The cells were then irradiated for 15 min with a light dose of 108 J/cm^2. The effects of ICG in the dark were evaluated by incubating the dishes, in darkness, with the same ICG doses as those used in the photoactivation experiments.

For RT experiments cells were irradiated with a dose of 2, 4 and 6 Gy with and without ICG. In the latter case the cells were incubated with ICG solution at 1.7 μg/ml or 3 μg/ml for 2 h, and the solution was replaced with fresh drug-free culture medium before ionizing irradiation.

For combined experiments cells were incubated with ICG solutions at 1.7 μg/ml or 3 μg/ml for 2 h. After incubation the solution was replaced with fresh drug-free culture medium. The cells were irradiated for 15 min at 805 nm with a light dose of 108 J/cm^2 and then irradiated for 15 min with 6 MeV photons at a dose of 2, 4, 6 Gy (PDT + RT). In the some experiments the treatment order was reversed (RT + PDT) with the same experimental conditions.

RESULTS

We found the uptake of ICG at 3 μg/ml after 2 h of drug exposure of 1.10 ± 0.22 ($\mu g/10^6$ cells) for PC-3 and 0.43 ± 0.09 ($\mu g/10^6$ cells) for EPN. These data represent mean values obtained in three independent experiments.

Figure 1 shows survival curves for the cell lines PC-3 and EPN, expressed in logarithmic scale as a function of the concentration of

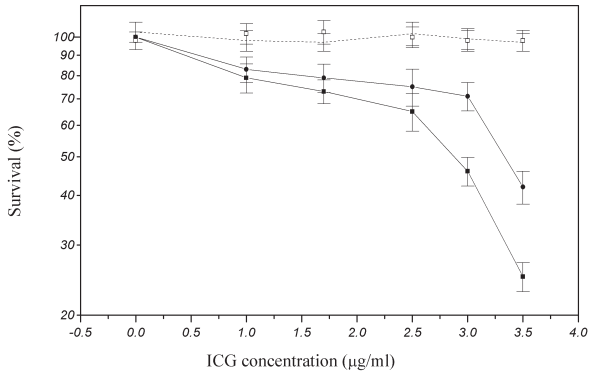

Figure 1. Cell survival of PC-3 cells (solid squares) and EPN cells (solid circles) *vs* the concentration of administered ICG and with a light dose of 108 J/cm^2 at 805 nm. Cell survival in the presence of ICG in the dark (PC-3, open squares; EPN, open circles) is also reported.

Bars give the ±S.D. of three measurements.

indocyanine green administered. The curves show that ICG at concentrations up to about 2.5 μg/ml causes a small dose-dependent

photocytotoxic effect similar in both cell lines tested. Experiments performed with ICG administration in the dark gave for both cell lines a survival of 100% at all concentrations used.

similar to that of EPN. In contrast, for ICG at 3.0 μg/ml and at ionizing radiation dose of 2 Gy the survival of PC-3 is significantly less than of the EPN cells. Assuming that the treatments have independent effects, we have

Table 1. Measured and calculated mean survival of PC-3 and EPN cells after different treatments with photodynamic therapy (PDT) and radiotherapy (RT).

Calculated survival is based on multiplication of single treatments.

Treatment	Measured mean survival		Calculated mean survival	
	EPN	PC3	EPN	PC3
PDT (1.7 μg/ml)	79	73	–	–
PDT (3 μg/ml)	71	46	–	–
RT (2 Gy)	72	71	–	–
RT (4 Gy)	38	36	–	–
RT (6 Gy)	20	18	–	–
PDT (1.7 μg/ml) + RT (2 Gy)	58	57	57	52
RT (2 Gy) + PDT (1.7 μg/ml)	61	55		
PDT (1.7 μg/ml) + RT (4 Gy)	31	27	30	26
RT (4 Gy) + PDT (1.7 μg/ml)	33	26		
PDT (1.7 μg/ml) + RT (6 Gy)	13	14	16	13
RT (6 Gy) + PDT (1.7 μg/ml)	12	13		
PDT (3 μg/ml) + RT (2 Gy)	55	35	51	33
RT (2 Gy) + PDT (3 μg/ml)	52	37		
PDT (3 μg/ml) + RT (4 Gy)	26	19	27	17
RT (4 Gy) + PDT (3 μg/ml)	23	21		
PDT (3 μg/ml) + RT (6 Gy)	12	7	14	8
RT (6 Gy) + PDT (3 μg/ml)	10	6		

The colony forming ability of PC-3 and EPN cells as a function of ionizing radiation dose is reported in Figs 2A–C. The cell survival decreases exponentially with the doses of radiation administered and does not depend on the presence or absence of ICG for either of the cell lines.

The combined effect of PDT and RT is shown in Table 1 with ICG at 1.7 μg/ml and at 3.0 μg/ml. For both ICG doses the cell survival is not significantly different irrespective of the order of the PDT and RT treatments. For ICG at 1.7 μg/ml the cell survival is very

calculated the EPN cellular survival values for combined treatments by multiplying cell survival values of the individual treatments. These values are reported in Table 1, and are close to the values of the combined treatments.

DISCUSSION

We have tested the photocytotoxic properties of ICG on prostate cancer cells PC-3, comparing them to normal EPN cells. The lack of

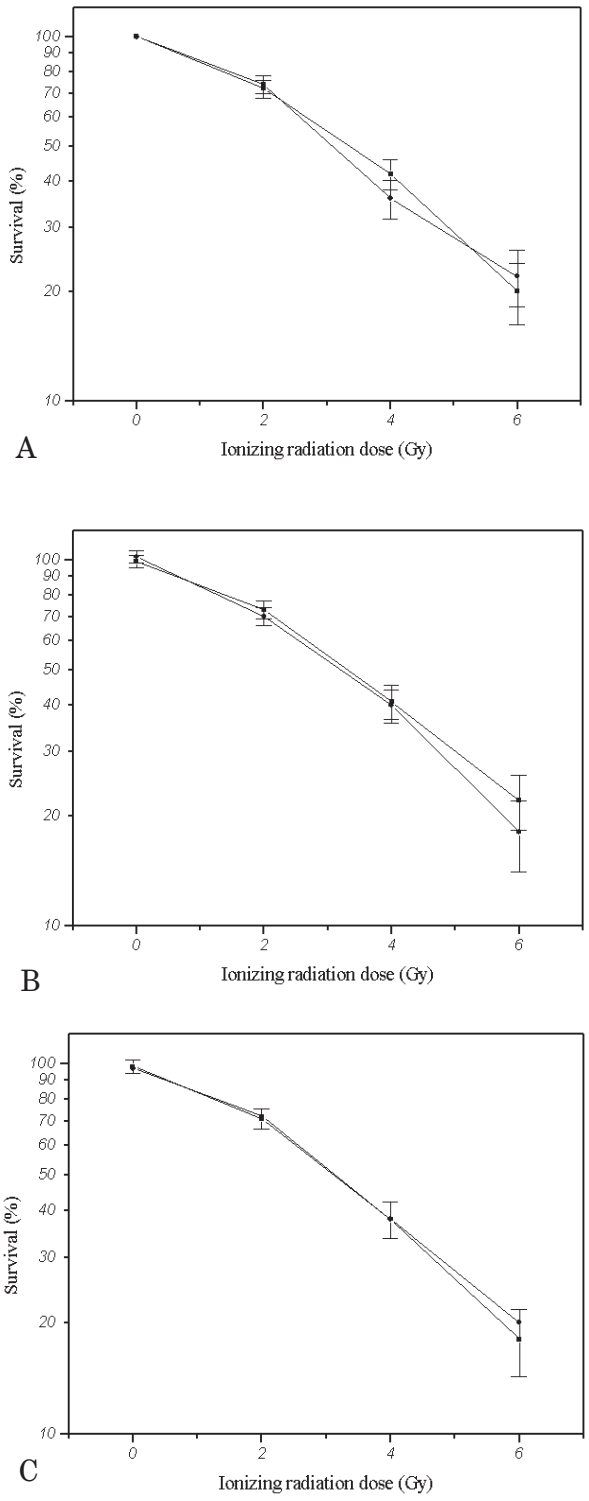

Figure 2. Cell survival of PC-3 (squares) and EPN (circles) lines.

A. In the absence of ICG as a function of ionizing radiation dose administered. B. In the presence of 1.7 μg/ml ICG, as a function of ionizing radiation dose administered. C. In the presence of 3.0 μg/ml ICG, as a function of ionizing radiation administered. Bars give the ±S.D. of three measurements.

an effect of ICG in the dark confirms that the decrease in survival occurring upon laser treatment was due solely to phototoxicity generated by ICG photoactivation. The results of ICG photoactivation show a flat course of survival up to 2.5 μg/ml ICG showing a small dose-dependent photocytotoxic effect for both cell lines (Fig. 1). At higher doses a significant difference between the survival curves of the two cell lines is present and the survival of PC-3 cells decreases by 35–40% in comparison with EPN cells.

The results obtained by ICG photoactivation reflect a difference in the photodynamic-induced effect that could depend on a dissimilar accumulation of ICG in the cells. In fact, the PC-3 cells display ICG uptake greater than the EPN cells by a factor of about 3.

We performed cell irradiation immediately after drug exposure because the drug does not stay in the cell for longer than 2–3 h: after 4 h in free medium, ICG concentration in both cell lines was below the sensitivity of our assay method (not shown).

The photodynamic action of ICG can be attributed to a type II process that yields singlet oxygen. The yield of triplet formation by S_1-T_1 intersystem-crossing for ICG is about 11–17% depending on the solvent used. Although this value is quite low, it is sufficient to consider ICG a phototoxic agent with type II reactions (Reindl *et al.*, 1997; Fickweiler *et al.*, 1997; Bäumler *et al.*, 1999). It has been shown that addition of sodium azide, a well-known singlet oxygen quencher, significantly reduces cell death while the administration of mannitol, a superoxide anion and free radical quencher, causes no variation in cell mortality after ICG-based PDT (Bäumler *et al.*, 1999).

The survival curves of PC-3 and EPN after RT treatment alone (Fig. 2) show the same dose-dependent effect for both cell lines, pointing out that the ionizing radiation treatment alone can not be considered selective because it produces equal killing of normal and neoplastic prostate cells. Moreover, cell sur-

vival as a function of the radiation dose does not depend on the presence of ICG. Therefore, ICG does not act as a radiosensitizer, unlike other drugs such as hematoporphyrin (Luksiene *et al.*, 1999) and Photofrin (Schaffer *et al.*, 2002).

The effects of the combined treatment for both cell lines were the same irrespective of the treatment sequence. Assuming that a synergic or antagonist action is absent in the combined treatment, the expected cell survival can be calculated by multiplying the cell survival values for the individual treatments (Table 1). The calculated cell survivals agree with those observed experimentally. Therefore, the combined effects of PDT and RT on the PC-3 and EPN cells point out that the two treatments act independently.

The combined PDT and RT treatment shows a difference between the two cell lines at 3 μg/ml ICG. On the other hand, no difference in survival is present with the radiotherapy only (Fig. 2A–C), whereas it is found with PDT only. Therefore the downward shift in the survival curve for the combined treatment can be attributed to a 20% reduction in clonogenesis between EPN and PC-3 as seen for the PDT. However, we think that the use of combined treatments could be advantageous. In fact, a 4 Gy dose is necessary to produce a cell survival of about 40% for both cell lines with RT only (Fig. 2A–C). In comparison PDT with 3 μg/ml ICG plus RT with half as large a dose of ionizing radiation produce the same result on PC-3 cells and, moreover, allow a greater cell survival of almost 60% for the normal cells (Table 1).

The absence of a synergic interaction between PDT and ionizing radiation agrees with the data of Ramakrishnan *et al.* (1990) and Bellnier and Dougherty (1986), whose studies show that only combined treatments with a time interval of about a minute cause synergic effects.

Moreover, the absence of a synergic effect on the PC-3 and EPN lines could be due to the cytotoxic damages induced by PDT and RT being located in different cellular sites (Ramakrishnan *et al.*, 1990). It is well known that photocytotoxic damage is caused by singlet oxygen acting on the cell membranes, while ionizing radiation induces main damage of DNA by direct ionization or free radicals.

CONCLUSIONS

Our results show that indocyanine green induces cell death following photoactivation. This effect is more pronounced in prostate cancer than in normal prostate cell, possibly due to stronger ICG uptake by the former.

ICG does not act as a radiosensitizer. The combined effects of PDT and RT on both cell lines show that the two treatments act independently.

Nevertheless, photodynamic therapy can be considered as a cytoreductive therapy prior to radiotherapy, enabling lowering of the radiation dose.

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