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Biological basis for chemo-radiotherapy interactions

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Abstract

For over 10 years, chemo-radiotherapeutic combinations have been used to treat locally advanced epithelial tumours. The rationale for these combinations relies on spatial cooperation or interaction between modalities. Interactions may take place (i) at the molecular level, with altered DNA repair or modification of the lesions induced by drugs or radiation, (ii) at the cellular level, notably through cytokinetic cooperation arising from differential sensitivity of the various compartments of the cell cycle to the drug or radiation, and (iii) at the tissue level, including reoxygenation, increased drug uptake or inhibition of repopulation or angiogenesis. Some mechanisms underlying interaction of radiation with *cis*-diammino-platinum (II) (*cis*-Pt), 5-fluoro-2′-deoxyuridine (5-FU), taxanes and gemcitabine are described. It is shown how various mechanisms including cell synchronisation and reoxygenation concur to paclitaxel-induced radiosensitisation. In the future, specific targeting of tumours, for example, with the epidermal growth factor receptor (EGFR) or angiogenesis inhibitors, should be achieved in order to increase the therapeutic index.

Keywords: Chemo-radiotherapy; Taxane; Gemcitabine

In 1979, Steel and his coworkers proposed a theoretical frame for the prediction of the outcome of combined treatments with cytotoxic chemotherapy and radiotherapy [1,2], including:

- *Spatial cooperation*. This concept was devised to mean that chemotherapy and radiotherapy are primarily designed to target metastatic disease and the primitive tumour site, respectively.
- *Additivity*, where it is intended that both modalities interact in a purely additive mode with regard to sterilisation of the target tumour.
- *Infra-additivity* (or protection), where the chemotherapeutic agent inhibits tumour regression (or protects normal tissue) by radiation.
- *Supra-additivity*, or enhanced tumour response, where the use of combinations ends in a more potent effect than expected from the summation of the effects for each agent applied alone. The term 'radiosensitisation' is not recommended unless the drug is devoid of any cytotoxic potential.

Where the drug carries protection against radiation damage to normal healthy tissues, this would theoretically allow the radiation dose to the tumour to be increased. In general, however, to obtain spatial cooperation or additivity, it is necessary to use chemo- and radiotherapy each at full doses or, at least, drugs at cytotoxic levels, yet there may be a few exceptions. For example, one European Organization for Research and Treatment of Cancer (EORTC) trial in lung cancer [3] where cisplatin was used at a sub-cytotoxic concentration with conventional radiotherapy, gave evidence of a substantial improvement in local control of the tumour. On the other hand, supra-additive interaction usually relies on molecular interaction between the drug and radiation. Numerous mechanisms of interactions have already been described and will be summarised below. This notwithstanding, in most instances the benefit of chemo-radiotherapy combinations in the clinic results from spatial cooperation or additivity.

1. Quantification of interactions between the drugs and radiations

If the cytotoxic response to the drug or radiation does not follow an exponential dose dependence, as is the

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case in most instances, the determination of the additivity status of the radiation–drug interaction cannot be reached directly from the inter-comparison of the survival curves. Moreover, data from combined treatment consist of three variables, namely the doses of the two agents and the resulting outcome. To overcome this problem, Steel and Peckham [1,4] proposed a method based on the isoeffect concept and relying on the construction of isobolograms for any given isoeffect. Actually, when the effect is fixed at a given value, e.g. at 10% survival, the system is reduced to two variables so that isobolograms for the chosen isoeffect may be constructed by plotting the doses of the two agents on separate axes.

The central concept in the isobologram method is the determination of the envelope of additivity delineated by 'mode I' and 'mode II' frontiers and within which all responses are deemed to be purely additive. Briefly, mode I and mode II curves are calculated by the addition of responses to each agent applied alone, using different regions of the experimental dose–effect relationships. These curves are assumed to represent complete independence of treatments (mode I) or exact complementation of the effect of one treatment by the other (mode II). The data points falling below (above) the envelope of additivity indicate supraadditive (infraadditive) interaction. Details of the calculations have been described elsewhere [5].

For a sufficient degree of significance, the construction of isobolograms requires quantitative measurements. This is one of the limits of the method. For example, healthy tissue response may not be readily incorporated in an isobologram. Moreover, isobologram analysis requires a careful, precise determination of the dose–effect relationship for each agent. This is because drug and ionising radiation produce dissimilar dose–response effects, i.e. the shapes of the survival curves are characteristically different. For the same reason, the additivity status may vary with the isoeffect considered, in such a way that supra-additive interaction, when it occurs, is usually more pronounced at a low degree of survival [5]. However, most preclinical additivity studies have come from *in vitro* experiments, and some caution should be taken in the transfer of these concepts to the clinic.

2. Molecular mechanisms of interaction

Antitumour drugs may provide various mechanisms of interaction with radiation including DNA repair inhibition, cell-cycle redistribution, or altered cytokinesis or apoptosis. The relative importance of these mechanisms has seldom been evaluated, even for *in vitro* studies. For each mechanisms, we shall provide examples with current antitumour drugs.

2.1. Additional damage or modification of radio-induced DNA damage

Ionising radiation induces a wide range of lesions in the DNA of target cells, including base damage, alkalilabile sites, single-strand breaks (SSB) and double-strand breaks (DSB). It has long been shown that these lesions are rapidly repaired, with the noticeable exception of DSB for which the $t_{1/2}$ for rejoining extends over 55 min or more in repair-proficient cells. Unrepaired DSB are consistently regarded as lethal lesions [6]. However, the contribution to induced cell kill of the oxidative stress associated with low-low energy transfer radiation (LET) and implying poly(ADP-ribose) polymerase for its repair, should not be underestimated [7].

Many chemotherapeutic drugs also target DNA, creating adducts, SSB or DSB. *cis*-diammino-platinum (II) (*cis*-Pt), for example, exert its cytoxic effect through the chelation of guanine residues, yielding monofunctional adducts and intrastrand or interstrand crosslinks. The bulk adducts are repaired through the excision repair pathway [8], but mismatch repair is also involved in the processing of cis-Pt adducts by the cell [9]. The possibility exists that the presence in close vicinity in DNA, of both a cis-Pt adduct and a radiation-induced SSB may result in a mutual impairment of proper repair (Fig. 1). This model is supported by calculations made to estimate the probability of interaction between cis-Pt adducts and radiation-induced SSB [10] and by experimental data as well [11].

Etoposide is a topoisomerase IIα poison. It acts through the stabilisation of abortive cleavable complexes and creates DNA DSB [12–14], notably in telomeres [15], and elicits maximum cytotoxicity in the S-phase of the cell cycle. A supra-additive interaction with radiation was demonstrated in concomitant exposure [5] and in the radiation-induced G2-block [16]. It has been proposed that this supra-additivity results from enhanced DNA damage in relation to chromatin conformational changes associated with active DNA repair [16].

2.2. Inhibition or alteration of radiation damage repair

Radiation recovery is currently considered as the main determinant of the fate of late responding tissue. Therefore, the use of potent inhibitors of repair in combination with radiation, is likely to result in late, unmanageable toxicities and should be studied in great detail for each drug to prevent the occurrence of deleterious combinations.

The body of data on radiation DNA repair mechanisms has grown considerably over the last 15 years. DSB are repaired through two main pathways, namely, non-homologous end-joining (NHEJ) and homologous recombination. Yet it is intrinsically error-prone, NHEJ

largely prevails in somatic mammalian cells. The main enzyme complex in NHEJ is the DNA-dependent serine/threonine protein kinase (DNA-PK). DNA-PK is formed from three subunits known as Ku70, Ku86 and DNA-PKcs. Deletion or mutation in any of these genes confers extreme sensitivity to radiation [17,18]. Like many other enzymes involved in DNA damage signalling or repair (ataxia-telangiectasia mutated (ATM), ataxia telangiectasis Rad3-related (ATR), FRAP, TRRAP), DNA-PKcs has a catalytic domain with high homology to phosphatidylinositol 3-kinase (PI3-K). DNA-PKcs may therefore be inhibited to near completion by PI3-K inhibitors, most notably wortmannin [19]. *In vitro,* wortmannin is not toxic *per se,* but it increases dramatically the cytotoxicity of radiation [20,21]. Unfortunately, PI3-K inhibitors are highly toxic *in vivo*.

DNA synthesis and DNA repair often share common pathways. This should provide a rationale for investigating the potential of DNA synthesis inhibitors in combination with radiation. In fact, the drugs that affect nucleoside and nucleotide metabolism are among the most effective and most widely used agents to sensitise tumour cells to radiation treatment, including fluoropyrimidines (5-fluoro-2′-deoxyuridine (5-FU), 9-β-D-arabinofuranosyl-2-fluoroadenine monophosphate (fludarabine), 2′,2′-difluoro-2′-deoxycytosine (gemcitabine)), thymidine analogues (5-bromo-2′-deoxyuridine (BrdUrd), 5-iodo-2′-deoxyuridine (IrdUrd)) and hydroxyurea.

5-FU inhibits thymidylate synthase and depletes the pool of nucleotide triphosphates, leading to cell cycle redistribution, DNA fragmentation and cell death [22]. The incorporation of 5-FU into DNA and RNA as fluoro-deoxyuridine, contributes to its cytotoxicity. 5-FU is a well known radiosensitiser. There is evidence suggesting that 5-FU-induced radiosensitisation relies on the 5-FU fraction which is incorporated into DNA [23,24]. As a matter of fact, radiosensitisation correlates with a decrease in the rate and extent of repair of radiation-induced DSB and the addition of thymidine to the culture medium reverses, in part, the susceptibility to radiation. Leucovorin, however, likely due to the enhanced trapping of fluoro-dUMP by thymidylate synthase, also acts as an enhancer of radiosensitisation by 5-FU, suggesting that an imbalanced deoxynucleoside triphosphate pool may be a major pathway of altered DNA repair. However, cell cycle redistribution following 5-FU exposure may also explain the enhanced radiation susceptibility [25].

Gemcitabine is a pyrimidine analogue with a wide range of activity against solid tumours. It acts to deplete the deoxynucleoside triphosphate pool and is incorporated into DNA, in the same way as 5-FU. A clear correlation between incorporation into DNA and cytotoxicity, with inhibition of DNA synthesis and probably DNA repair, has been demonstrated [26]. Gemcitabine has been found to exert a major radiosensitising effect in colon, pancreatic and squamous cell carcinoma cell lines in relation to the S-phase cell content [27–29]. The effect was observed for relatively low drug concentrations, reached a maximum of 24 h after the onset of drug exposure [30] and persisted for more than 48 h after contact with drug [31]. Depletion of the deoxynucleoside triphosphate pool contributes to the enhanced radiation susceptibility [27,30], but no increase in the incidence or repair of DNA strand breaks by gemcitabine could be evidenced in an analysis of $\geqslant$200 kbp fragments by pulsed field gel electrophoresis [32,33]. This seems to rule out any direct inhibition

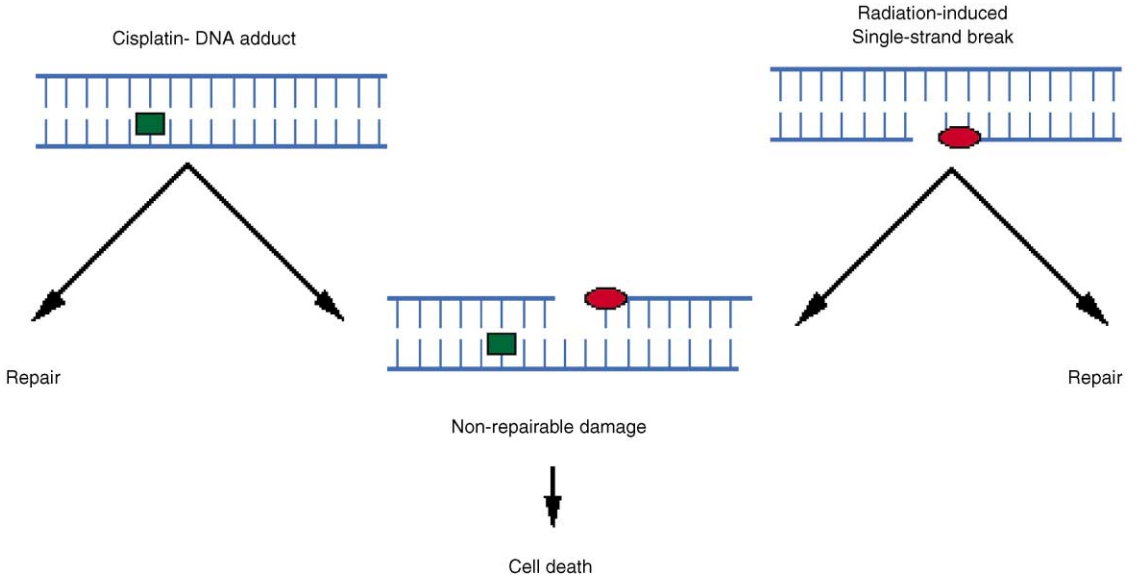

Fig. 1. Model showing response to DNA damage. A cisplatin DNA adduct or a radiation-induced single-strand break is likely to be repairable. In contrast, when both are observed, there may be a mutual impairment of proper repair resulting in the death of the cell.

of DNA repair pathways. In spite of this, major toxicity has been reported during the preliminary clinical trials of the gemcitabine–radiation combination [34], such that it has been recommended that this association should not be used beyond carefully designed clinical trials.

BrdUrd and IdUrd readily substitute for deoxythymidine and incorporate into DNA, thus inducing potent radiosensitisation with enhanced DNA damage and decreased DNA repair [35]. BrdUrd and IdUrd are believed to act both through an enhanced yield of radical damage at the C-5 position of the pyrimidine ring [36–38]. The magnitude of the effect in terms of survival consistently correlates with the amount of drug incorporated into DNA [39]. However, halopyrimidines can hardly be used as adjuvants to radiotherapy in view of their general toxicity.

3. Interactions at the cellular level

3.1. Cytokinetic cooperation

It has long been known that radiosensitivity changes with the progression of cells through the cell cycle. The S phase is most radioresistant, and the G2-M phase is usually most radiosensitive [40,41]. For this reason, a large increase in radiation susceptibility is observed as proliferating cells are exposed in close temporal proximity with radiation, to drugs which specifically kill cells in S phase. This is the case for the camptothecin and camptothecin analogues acting as topoisomerase I poisons [42], and possibly also for gemcitabine. It is best in these instances to consider that the effect proceeds from cytokinetic cooperation rather than radiosensitisation, since the drug is inactive against non-S phase cells and does not affect the radiation response among survivors [42–45]. Moreover, it was shown that the camptothecin–radiation interaction represents a pure mode II additivity according to the terminology used for isobolograms [42]. In contrast, topoisomerase I-targeting agents are able to alter the radiation response in confluent-arrested cells, possibly through interaction with DNA repair [46–48].

3.2. Synchronisation

Maximum radiosensitivity is usually observed in the G2-M phase of the cell cycle. Therefore, synchronisation of the cells in G2-M, if it occurs, is expected to elicit the maximum response to radiotherapy. For a few years, this was proposed as a rationale for the use of paclitaxel in conjunction with radiotherapy.

Paclitaxel and docetaxel bind with a high affinity to microtubules and alter their dynamics [49–54]. At high, cytotoxic doses, both drugs were shown to inhibit the formation of the mitotic spindle and consistently block the progression of cells in mitosis, between prophase and metaphase [55,56]. In fact, both drugs act through disruption of the centrosome network, thus inducing faulty mitosis and cytokinesis [57]. Pioneering studies of radiation interaction with paclitaxel indicated that increased radiosensitivity occurred at the time of the G2-M block [58–62]. However, further studies showed that enhanced radiation cell kill by taxanes does not work in all cell lines, and it was recognised that prometaphase arrest upon prolonged contact with drugs, if it occurs, may not be a sufficient condition for increased radiation sensitivity [63–66]. This was confirmed through isobologram analysis [67]. Moreover, in some cell lines paclitaxel and docetaxel at low doses may induce protection against radiation-induced cell kill, presumably through alteration of signal transduction pathways [66,68]. It should be mentioned at this stage that paclitaxel and docetaxel elicit different time-dependent interactions with radiation, due to different rates of excretion and cell-cycle specificity [69].

In vitro radiosensitisation of cells by fludarabine [70] and 5-fluoro-2′-deoxyuridine (FdUrd) [25] has also been proposed to proceed from the accumulation of cells in a radiosensitive compartment of the cell cycle.

Whether cell cycle redistribution might be used to increase the tumour response to radiotherapy is open to discussion [71]. The usefulness of this approach can be questioned for many reasons. First of all, tumours are heterogeneous, with an uncontrolled amount of cells in the quiescent (G0) state, and the possibility of induced cell synchronisation by antimetabolites or DNA polymerase inhibitors in humans is extremely limited. Second, as normal tissue surrounding the tumour may also be a target for cell synchronisation, such synchronisation may not necessarily result in an increased therapeutix index. Furthermore, conflicting data have been reported as to whether G2-M cells are more prone to apoptosis than cells in other phases of the cell cycle [72].

3.3. Promotion of apoptosis

Cells may undergo lysis or lose reproductive ability through various unscheduled (immediate, mitotic and delayed cell death) or programmed (apoptosis and senescence) mechanisms. Mitotic cell death, involving abortive mitosis and oncosis (improperly referred to as necrosis), is by far the most frequent mode of cell death in epithelial tumour cells. Recent developments have shed light on the basic mechanisms of mitotic cell death, such as defects in the control of centrosome replication [73,74]. However, for the last decade interest has turned mainly to apoptosis, simply because it is most amenable to studies based on molecular biology and genetics.

Lymphocytes, thymocytes, prostate cells, salivary acini, endothelial cells and intestinal crypts, are the most sensitive cells to apoptosis in response to DSB induction,

oxidative stress or hypoxia, ceramide, contact with some cytokines or deprivation of growth factors. The p53 protein, due for the most part to its role in increased (decreased) transcription of the pro-apoptotic (anti-apoptotic) mitochondrial proteins Bax (Bcl-2), is mandatory for radiation-induced apoptosis (for a review, see Ref. [75]). Conversely, *TP53* mutation or deletion reportedly promotes apoptosis after paclitaxel treatment [76]. A kind of a cooperation depending on the p53 status and the cytotoxic agent could thus be proposed, yet this question is still very much a matter of debate [77].

Apoptosis undoubtedly plays a major role in cell killing by radiation or DNA-nicking drugs applied alone, e.g. for topoisomerase I- or II-targeting agents. However, evidence in favour of increased apoptosis as a general mechanism to account for increased response to combined treatment, is not firmly established. For example, increased apoptosis might account for radiosensitisation by gemcitabine in cell lines that are prone to radiation-induced apoptosis [78]; however, a radiosensitising effect of gemcitabine is also observed in apoptosis-resistant cells.

4. The search for tumour specificity

The way to a more efficient anticancer treatment would be to target tumours with treatments eliciting minimal response in surrounding, dose-limiting normal tissue. Radiotherapy takes advantage of differential sublethal damage repair in tumours versus normal tissue. Unfortunately, there is no convincing evidence to show that normal tissue sparing is retained when the chemo-radiotherapy combination is used, and, in fact, randomised trials for the appreciation of the late toxicity of chemo-radiotherapy in randomised trials, are still lacking.

Such studies should be encouraged taking into account some well-known properties of solid tumours that make them resistant to treatment. In particular, (i) tumours are frequently hypoxic, in relation to defective angiogenesis, and respond poorly to radiotherapy; (ii) some tumours contain a large proportion of quiescent cells, which are usually resistant to chemotherapy; (iii) in contrast, target tumours for radiotherapy are often rapidly proliferating, with a high amount of radioresistant S-phase cells; (iv) epithelial tumour cells overexpress growth factors receptors, or may grow independently of growth factors; (v) tumour cells are mutated or deleted for genes involved in genome maintenance or cell cycle control.

4.1. Reoxygenation and tumour shrinkage

A reduction in tumour volume after treatment with one modality may result in an improved blood supply to the tumour, leading to reoxygenation and increased radiosensitivity and chemosensitivity. This was clearly demonstrated by Milas and colleagues [79] in a human tumour xenograft treated with paclitaxel prior to irradiation. The fraction of hypoxic cells was measured by micro-electrodes introduced in the tumour before and after drug treatment. The median pO_2 values in this experiment were 6.2 Tor in the untreated tumours, and increased to 10.5 and 31.2 Tor at 24 and 48 h after paclitaxel treatment, respectively. Reoxygenation correlated with an increased radiation response, irrespective of whether cells accumulated in G2-M or not [79]. Similar reoxygenation and radiosensitisation has been reported following gemcitabine treatment [80].

Fractionated irradiation, through a reduction of the tumour volume, may also increase the tumour blood flow and facilitate drug access to the tumour. Indeed, it has been demonstrated that radiation increases the uptake of some drugs, like carboplatin and 5-FU [81,82].

4.2. Inhibition of tumour proliferation

Tumour repopulation is often invoked to account for the failure of radiotherapy [83]. Although the mechanisms involved in tumour regrowth are not completely understood, the role of growth factors is likely to be of major importance [84].

Modulation of tumour proliferation may be achieved by epidermal growth factor receptor (EGFR) inhibition, either with a monoclonal antibody directed against the receptor (C225 mAb) or through the inactivation of the tyrosine-kinase activity of EGFR [85,86]. Preclinical studies have demonstrated the ability of C225 to enhance *in vitro* radiosensitivity. Several mechanisms have been proposed to explain this observation, including inhibition of cell proliferation, of DNA damage repair [87], of tumour angiogenesis [88] or, in contrast, promotion of radiation-induced apoptosis. The inhibition of EGFR seems to be a promising way to increase the cytotoxic effect of radiation. However, due to the fact that repopulation of normal, rapidly responding tissues is the rule after irradiation, acute toxicity may be feared. Careful phase I studies with EGFR inhibitors in combination with radiation, should therefore be planned before any phase II or III studies.

4.3. Inhibition of angiogenesis

Angiogenesis is essential for tumour growth. Consistently, the search for compounds endowed with anti-neoangiogenic activity is flourishing, including angiostatin, combretastatin, flavone derivatives or kinase inhibitors. The combination of angiostatin and irradiation was found to have a major antitumoral effect in a human tumour xenograft model [89]. This synergy

may arise from a cytotoxic effect on endothelial cells. Others compounds, such as TNP-470, may also elicit a radiosensitising potential [90].

4.4. Specificity for tumour tissues

Another way to increased specificity against tumour tissue is to make use of drugs able to target a particular organ. For instance, estramustine has proven highly specific for prostate tissues and may act as a radiosensitiser [91,92]. Clinical trials have started with this molecule in prostate cancer, in the hopes that a specific radiosensitisation of these tumours may be obtained [93].

5. Conclusion

Concomitant chemo-radiotherapy turns out to be a widely accepted approach for the treatment of locally advanced epithelial carcinomas. Laboratory studies may contribute to drug development and help the physician in three different ways.

First, in selecting drugs which present a potential for radiosensitisation or for additive cytotoxicity. This requires measuring the strength of the effect, and determining whether the drugs are able or not to inhibit the repair of radiation-induced damage. It should be kept in mind that repair inhibition may lead to acute hypertoxicity and treatment failure, as earlier observed with bleomycin and more recently with gemcitabine. Therefore, great care should be taken in the design of the protocols for combined treatments assays, as long as pronounced radiosensitisation is observed *in vitro*.

Second, by improving the time schedule of the chemo-radiotherapy regimens and the mode of drug administration. Should the drug be given concomitantly, before or after irradiation, as a bolus treatment or as a continuous infusion? Studies performed with 5-FU provide a classical example of the benefit of such studies. Indeed, it has been shown that 5-FU must be given after irradiation, and for at least 24 h to provide a more-than-additive interaction [94]. Moreover, continuous infusion of 5-FU offers a better radiosensitisation than bolus administration [95], yet it may entail enhanced toxicity. These data have been confirmed by a randomised trial in rectal carcinoma [96].

Last, but not least, the evaluation of new cytostatic, non-cytotoxic drugs, e.g. farnesyl transferase or cyclin-dependent kinase inhibitors, should always been done in combination with other drugs or radiation. This may help in the understanding of the mode of action of the drugs *in vivo,* provide new targets for drug development, and avoid some of the pitfalls encountered in the transfer to the clinic of bench research in molecular and cellular biology.

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