MECHANISM OF OXYGEN SENSITIZATION IN RADIOBIOLOGY — A REVIEW

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INTRODUCTION

Thomlinson and Gray(1) in their histological examination of human lung tumour sections, observed that the onset of necrosis in the tumours coincided with the depletion of oxygen supply from microcapillaries at distances greater than 150 — 200μ. The authors inferred that areas of necrosis occured as a result of oxygen depletion caused by oxygen metabolism by the intervening tumour cells. The radiobiological resistant cells have a very low level of oxygen content although sufficient to keep the cells alive but low enough to prevent cell division. On irradiation, the aerated cells are killed but the resistant cells survive. The nutrient supply can then reach the latter cells. These cells become oxygenated and can then proliferate.

The importance of the oxygen effect in the treatment of tumours by radiotherapy has led radiobiologists to study the basic mechanism of radiation damage and the oxygen effect in vitro. Here various radiobiological tools such as varying LET radiation, fast response methods, etc. are used in order to understand the exact mechanism of radiosensitization by oxygen.

THE OXYGEN EFFECT

Why the oxygen enhancement ratio (OER) of cell-killing decreased with increasing linear energy transfer (LET) has long been the concern of the radiobiologist. In the early days where the direct action of radiation was first considered, the multihit/multitarget models were used in an attempt to solve the problem. When radiolysis of water played a role in the fixation of damage, it was proposed that hydrogen peroxide produced in the tracks of densely ionising radiation fixed the damage. However, it was found that hydrogen peroxide production in chemical systems did not increase rapidly in the LET range where the OER decreased rapidly.

Alper(2) proposed the "interacting radicals" hypothesis where radicals formed close together by the densely ionising radiation have a greater probability of interacting with each other — thus the damage can be fixed without the intervention of oxygen. The concept of direct deposition of energy in the critical target/s was again considered. Howard-Flanders and Alper(3) expressed the dependence of radiation sensitivity, R, for low LET radiation, on the oxygen concentration (O_2) by the formula:-

$$R = \frac{K + m(O_2)}{K + (O_2)} \qquad (1)$$

where m = maximum OER
K = constant

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Alper's hypothesis(3) was challenged by Neary(4) in 1965 who proposed the oxygen-in-the track hypothesis. Here the oxygen produced in the tracks of densely ionising radiation influence the dose-effect survival curves performed in anoxic conditions. The two hypotheses were put to test using the data from **Shigella flexneri** and **Chlamydomonas reinhardii.** The K value in equation (1) increased with increasing LET thus supporting the oxygen-in-the track hypothesis (5). Chemical systems were also used to measure the amount of oxygen produced by densely ionising radiation and the data obtained supported this hypothesis (6). Barendsen (7) argued that if oxygen is produced, a physico-chemical phenomena, then the decrease in OER with increase in LET should be independent of the cell lines used.

The LET concept and the track-segment theory were developed based on microdosimetry in the attempts to explain the oxygen effect. Based on the concept of Lea and Catchside (8) that several ionisations were required to inactivate a single target, Howard-Flanders (9) postulated that two mechanisms were involved in the production of the observed biological effects. The first mechanism was a single-hit event which depended upon the presence of oxygen. The second mechanism was oxygen independent and was based on a multi-hit event. Barendsen (7) employed the second mechanism to explain within a critical distance t. In anoxia either n must be larger by 30-35% with t remaining constant or n remains the same whilst the distance t is smaller by 30-35%. This was based on the observation that cross-sections for cellular inactivation depended on the LET and the curves for the aerobic and anoxic cases were displaced by approximately a constant factor (10, 11). The dose-modifying factor of the chemical lesions formed is independent of the radiation quality and the dependence of OER on LET would follow the dependence of the inactivation cross-section on LET. However, the hypothesis failed to explain the experimentally observed rapid decrease in OER in the LET region of 100 KeV/um.

Kellerer and Rossi (12) observed that a rapid increase in the inactivation cross-section in the 100 KeV/um region in the data of Barendsen et al (10) was significant in hypoxia. Hence a greater number of sublesions would be formed and this would account for the rapid experimental decrease in OER in this LET region.

SPORES

Dry spores are regarded as living cells since they are capable of undergoing similar biological reproductive processes as other bacterial systems. However, spores can be biologically inert under some conditions i.e. there is no metabolic activity at the time of irradiation (13, 14). Hence the initial radiation events studied are due to a physicochemical rather than a biochemical process. Since radiation lethal events in bacterial and mammalian cells occur on such a short time scale, early studies on this system were hampered by physical limitations e.g. the shortest time available in rapid-transfer experiments was 20 ms (15). Hence dried spores provided an alternative system for the study of radiation lethal events as the damaging species become more long-lived in the dry state.

The early studies on dried spores with radiation led to the recognition of three distinct components of damage termed as Class I, Class II and Class III damage (13). Class I damage is independent of oxygen whilst the other classes require the presence of oxygen to fix the damage. Class II damage is only observed when oxygen is present at the time of irradiation — a short-lived species. Class III damage has a long post-irradiation lifetime and is a radiation-induced free radical (16). Since these classes of damage were first observed in dried spores, the next step was to establish whether these kinds of damage operate in wet spores.

In water-saturated spores, there appeared only one oxygen effect (14). However, the authors proposed that the two oxygen effects may be operating in the system but were not distinguished using the available experimental techniques. Tallentire and co-workers (17) resolved the two oxygen effects on a concentration basis and this was later observed by Ewing and Powers (18).

The oxygen-dependent species were also resolved on a time basis (19, 20), in wet spores using a pulsed-irradiation and rapid-mixing technique. These species were resolved only when irradiation was performed in anoxia and when oxygen was introduced at known time intervals. The lifetimes of these potentially-damaging species were 9s and 120s. However, Weiss and Santomasso (21) observed only the decay of one oxygen-sensitive species (half-life of 10.5s or 7.4s depending on the radiation dose used).

In the presence of oxygen only the short-lived sensitive species (half-life of 9s) was observed (19, 20). These authors proposed that the difference in the lifetimes of damaging species in spores compared to those in vegetative bacteria and mammalian cells could be due to the interior of spores being much less hydrated. If the degree of hydration is similar then one might expect shorter lifetimes comparable to those in the cells.

FAST-RESPONSE METHODS

Since lifetimes of target/s in bacterial and mammalian cells were in the millisecond timescale, it was necessary to develop fast techniques of irradiation. At present there exist three such techniques. They are:

(i) single and double-pulse method
(ii) gas-explosion method
(iii) rapid-mix method

In the double-pulse method, the first pulse depletes the intracellular oxygen and the second pulse is then given at known time intervals; between 10^{-6}s to 30s by which time a given amount of oxygen should have diffused into the cells. Using this method the upper limit of lifetime of oxygen-sensitive species were established in different biological species. The values were tabulated in table I.

Table I

Biological System	Upper Limit of Lifetime of Oxygen-Sensitive Species	
E. Coli B/r	10^{-4}s	(22)
Serratia marcescens	10^{-4}s	(23)
CHO cells	2 ms	(24)

The difference in lifetimes of reactive species between bacterial and mammalian cells was attributed to the difference in cellular size, as the measurement of lifetimes was based on assumptions concerning the diffusion of oxygen to their respective critical sites.

The gas-explosion method is based on fast-mixing and irradiation with a single high intensity pulse (25). Here the cell system is irradiated with a single short pulse at either pre- or post-arrival of gaseous oxygen at present time intervals (time resolution — 100μs). The effect of time delay between irradiation and oxygen exposure on the surviving fraction can be used to obtain information on the repair kinetics of oxygen dependent damaging species.

In **Serratia marcescens,** two types of oxygen-dependent damage were identified (26). The fast component (70% of total damage) which represents fast repair occurs at less than 1.5 ms. The slow component, representing slow repair, occurs at times greater than 1.5 ms. In Chinese hamster V79-379A cells, only one type of oxygen-

dependent damage has been resolved with this technique (27). Full sensitization was observed between 1-2 ms.

In the double-pulse and gas-explosion methods, the dose-modifying substances had to be volatile e.g. oxygen. In the liquid-flow rapid-mix method, water soluble dose-modifying agents can be used in the study of dose-modification of bacterial and mammalian cells.

Using this method, Shenoy and co-workers (28) observed that oxygen sensitization in Chinese hamster cells could be resolved into two distinct components. The authors postulated that the two components could indicate either two oxygen-sensitive species of differing lifetimes or two reactive sites i.e. the nuclear membrane and the chromosomal DNA. In a later study on DNA single-strand breaks, no correlation was observed between strand breaks and cell survival in regard to their time dependence (29). However, Whillans et al (30) using CHO cells and Watts et al (31) and Kandaiya et al (32) using Chinese hamster cells could not repeat the data of Shenoy et al (28); full sensitisation by oxygen occurred within 5 ms.

THE CELL CYCLE EFFECT

Apart from the large diffenence in the radiosensitivities of oxygenated and hypoxic cells, there is also a large variation in the radiosensitivity of mammalian cells in terms of their cell age. In an asynchronous population the cells that survive a radiation dose are largely in the radioresistant phases of the cell cycle. The cell cycle study is another means of helping to identify the lethal lesions involved in radiation damage.

The general characteristics of cell age-response to low LET radiation indicate a resistant period in the G1 stage followed by a gradually increasing sensitivity towards the end of G1 and the begginning of S phase (i.e. G1/S stage). Through the S stage, there is a rapid increase in resistance with maximum resistance at late S. Then there is a rapid increase in sensitivity during G2 followed by maximum sensitivity for cells in mitosis (33, 34). The difference in the radiation response through the cell cycle has also been investigated as a function of LET and by the use of biochemical methods.

Synchronised mammalian cells exposed to radiation with a range of LET from 10 to 200 KeV/um follow a general trend in their cell cycle response. For low and intermediate LETS (48-190 KeV/um), there is an age dependent variation in cell killing but a gradual reduction in radiation sensitivity in cell cycle with increasing LET (35, 36, 37, 38). For very high LET radiation, the cell is equally sensitive at all stages of the cell cycle.

Low LET age response cannot be explained simply in terms of DNA content in cells (39, 40, 41). In the G2 stage, the DNA content is the same as in the S stage but there is a marked difference in sensitivity towards radiation. The hypothesis that nuclear size is a determinant of cellular sensitivity has failed to explain cell cycle variation. Bird et al (41) found the following data shown in Table 2 for cells in G1/S and late S phases when exposed to 170 keV/um radiation.

Table 2: THE CROSS-SECTIONS MEASURED AT THE TWO STAGES OF THE CELL CYCLE

	Cell Stage	
	Late S	G1/S
Inactivation cross-section	19μm^2	38μm^2
Cell nucleus cross-section	222μm^2	165μm^2

The authors proposed that although the DNA material almost doubles from G1/S transition to late S phase, the lower inactivation cross-section for S phase suggests a redundancy of sites or a much greater capacity to repair high LET damage then cells in G1/S phase.

Another index, that for chromosomal aberrations, was used to measure the variation in radiosensitivity in age response (42, 43). Dewey and co-workers (43) proposed that spatial variation in the chromatin fibres was responsible for the cell cycle effect. However, the increase in radiosensitivity at the G1/S border did not correspond to an increase in chromosome aberrations.

Sinclair (44) suggested that another property, the Q factor, governed the radiation response. From evidence based mainly on the variation in the degree of protection by cysteamine (45) and the degree of dose modification by NEM (44, 46), he showed that the Q factor must be related to the presence of intracellular thiol groups. Protection with cysteamine was highest during the most sensitive phase of the cell cycle where the concentration of thiol groups is the lowest, and least during the most resistant phase of the cell cycle where the concentration of the thiol groups is the highest. This would also explain the cyclic variation in the lethal response to ionising radiation by different cell-lines.

Radiation Sensitisers

Radiation sensitisers can be classified into 5 main categories (47):-

1. Sulphydryl — binding agents
2. Radiation — induced cytotoxicity
3. Structural incorporation of DNA analogues
4. Modification of cellular processes
5. Hypoxic cell sensitisers

In the oxygen effect in radiobiology, final group of sensitisers is of great interest. As the name indicates, these sensitisers sensitise hypoxic cells without affecting the aerated cells.

Hypoxic cell sensitisers can be classified into three sub-groups:

a. Nitroxyl stable free radical agents
b. Membrane — specific drugs
c. Electron — affinic agents

The electron-affinic agents being oxygen-mimetic agents provide another means of studying the oxygen effect indirectly in biological systems. These agents could be of clinical relevance even though their sensitising efficiencies are less than oxygen but their metabolic lifetimes in vivo (unlike oxygen) are long. This would then allow time for the drugs to diffuse out from tumour blood supply and reach the hypoxic cells.

The term "electron-affinic" was used as the efficiencies of sensitisation by these drugs are found to correlate with the electron-affinities of the compounds (48, 49, 50). Adams et al (51) fitted a multiple regression analysis between the E_7^1 (one electron reduction potential) and the sensitising efficiency of hypoxic Chinese hamster cells in vitro for 44 nitroaromatic and nitrohetrocyclic compounds. They concluded that the sensitising efficiency in vitro was strongly dependent on the reduction potential whilst the partition coefficient was unimportant. However, Anderson and Patel (52) using seven compounds of similar electron-affinity as misonidazole but varying partition coefficients between 0.11 and 120 observed a parabolic dependence of sensitisation on partition coefficient in anoxic bacterial cells. In the study of Adams and co-workers (51) 32 out of the 38 nitroaromatic compounds had partition coefficients <3.5 and the effect of compounds having higher partition coefficients would be masked especially when there is a threshold dependence (52).

In the search of better radiosensitisers, 2-nitroimidazoles substituted in the one position by side chains of varying lengths of carbon atoms but almost similar electron-affinity were studied (53). Here a chain

length containing 5 carbon atoms was the most effective sensitiser. A simple 4-nitroimidazole, a poor radiosensitiser when substituted at the 5 position by sulphur-containing groups proved to be very effective in the radiosensitisation of hypoxic Chinese hamster cells (54). Using electron-affinity as the basis for efficiency of radiosensitisation, other parameters can be varied to develop better radiosensitisers.

A COMPARISON OF ACTION OF HYPOXIC CELL RADIOSENSITISERS WITH THAT OF OXYGEN IN VITRO

In the 1960s reports of hypoxic cell radiosensitisation were confined to bacterial systems. The search was for efficient radiosensitisers in mammalian cell systems which could be applicable in the clinic. Successful sensitisation of mammalian cells was achieved with paranitroacetophenone (PNAP) (55). p-Nitro-3-dimethylamino-propiophenone HC1 (NDPP) a more soluble compound than PNAP, was an efficient radiosensitiser in bacterial and mammalian cells. In a detailed study in vitro NDPP was found to be a dual action type sensitiser (56). One was an electron-affinic component whilst the other, a biochemical component involved the suppression of endogenous sulphydryl groups.

Following the observation on the radiosensitising ability of analogues of nitrobenzenes, Chapman and co-workers (57) found nitrofurans, used as antibacterials clinically, to be potent hypoxic radiosensitisers. In vivo these drugs were rather disappointing due to their metabolic instability and toxicity.

At this time another class of nitroheterocyclic, the nitroimidazoles appeared to be more promising. Metronidazole or flagyl, a 4-nitroimidazole, and misonidazole, a 2-nitroimidazole, were studied extensively in vitro in relation to the oxygen effect. Sensitisation by flagyl (58) and misonidazole (59) was found to be independent of cell cycle and to be unaffected serum protein.

These drugs mimic most of the oxygen effect though there are some differences. Hall and Roizin-Towle (60) observed that misonidazole could not repair sublethal damage as demonstrated in their split-dose experiments. The criterion that fast free radicals were involved in senstisation was satisfied for PNAP, NDPP, misonidazole and metronidazole (61, 62). Adams et al (61) reported that PNAP appeared to mimic the "fast component" of the oxygen effect. From experiments under stationary-state conditions McNally and de Ronde (63) concluded that PNAP mimicked only one component of the oxygen effect.

Misonidazole seemed to compete effectively for similar lesions as oxygen (63) i.e. misonidazole was inferred to act on the two components of oxygen observed on the time resolved basis using the rapid-mix apparatus (28). Using the gas-explosion method, Michael and co-workers (26) reported that misonidazole appeared to act on an even shorter-lived form of oxygen-dependent damage. Millar and co-workers (64) observed two concentration components of misonidazole sensitisation which was similar to the two component oxygen sensitisation resolved on concentration basis for their cell line (65).

Studies on whether electron-affinic radiosensitisers compete for similar lesion/s as oxygen have been carried out mostly in combination studies under stationary state conditions. Fast response methods and the use of varying LET are other means of comparing the mechanism of these sensitisers with that of oxygen.

PROPOSED HYPOTHETICAL MECHANISMS OF RADIOSENSITISATION

The mechanism which results in loss of biological activity in the irradiated cells takes place instantaneously within a millisecond time scale as illustrated by the use of fast response techniques. This can occur by indirect and direct effects of ionising radiations. In the indirect effect, the events are mediated through diffusing free radicals, mainly those produced by action of radiation on intracellular water, e.g. OH, H and e^-_{aq}. In the direct effect, the events are produced by the action of radiation directly on the target molecules without any chemical intermediates.

(a) Indirect Effect of radiation damage

(i) The electron — transport model (48)

In the proposed model, the sensitising agents act as electron transport agents. Since there are many electron scavenging molecules in the cell, the electron-affinity of the sensitiser must be greater than the former molecules. The sensitiser-electron complex formed is able to diffuse to the target site/s further than the e^-_{aq} as the electron delocalised in the sensitiser is quasi-stable and has a longer lifetime than e^-_{aq}. The target which has an even higher electron-affinity captures the electron from the sensitiser. However, this model was abandoned when Blok and co-workers (66) showed that $O_2^{\cdot-}$ is relatively unreactive and does not inactivate the DNA.

(ii) Electron transfer and/or sensitiser adduct

The reactions of OH are oxidative whilst those of e^-_{aq} and H are reductive. From the considerable information from pulse radiolysis data on model compounds of DNA, it was proposed that radicals produced from the attack of primary species with the target molecules may react with the sensitiser. Here either an electron-transfer from the radical to the sensitiser or a senitiser adduct formed would inactivate the cell.

(iii) Sequestration of aqueous electron (67)

In the radiolysis of water e^-_{aq}, OH and H are produced. The radiosensitiser sequesters e^-_{aq} which would otherwise remove OH.

i.e. $e^-_{aq} + OH \rightarrow {}^-OH$

Good radiosensitisers would react well with e^-_{aq} but poorly with OH which attacks the target molecule.

(b) Direct effect of radiation damage

(i) The oxygen-fixation hypothesis

Alexander and Charlesby (68) postulated that energy absorbed in the target molecule will result in unalterable damage. In their experiments using solid polymers they showed that protection is also possible against direct action of radiation. To explain the action of dose-modifying agents such as oxygen and chemical protectors by direct action, Alper (2) postulated that after the passage of an ionising particle the target is left in a highly reactive state and will be involved in chemical reactions within a very short time — less than a ms or μs. The term "metionic reaction" was proposed. This reaction could involve recapture of an electron, reaction with neighbouring unionised molecules, etc.

(ii) Model proposed by Adams and Cooke (50)

From the failure of the electron transport model, it was realised that the electron was transferred to and not from the sensitiser. The radiation deposited in the target gives rise to an ion-pair. The electron ejected in this process, migrates to electron deficient centres in the target molecule. The damage produced depends

on the competition between the number of electron traps and the efficiency of charge neutralisation. If a sensitiser of higher electron-affinity is present as a complex or as a free molecule, the electron transfer from the ionised molecule to the sensitiser would occur. Irreversible electron transfer to the sensitiser would lead to an increase in the number of free radicals at the target.

The direct action of radiation on DNA is likely to be important since the chromosomes contain at least 30% solid material. Hence an appreciable fraction of energy would be released here (69). That DNA is capable of conducting electrons is shown in the luminescent study of BudR incorporation into dry DNA (70). Adams and Willson (71) also found that electron transfer reactions can occur rapidly from radical anions of simple nucleic acid derivates to brominated analogues diffusing freely in solution.

CONCLUSION

The oxygen effect has been studied both directly and indirectly in biological systems. There is no doubt that oxygen sensitisation occurs by fast free radical reactions. However the sites of action and the reaction mechanism has not been resolved yet. Once the mechanism can be understood in cells, then this would lead to effective means of treating hypoxic tumour cells.

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