Anhang/Appendix T2

DISRUPTION OF CELL-CELL INTERACTION AND CONSEQUENCES TOWARDS RADIOSENSITIVITY

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Effects of Intercellular Contact.

It has been recognized for some time that drug resistance and radioresistance can develop when cells are part of a larger assembly, often dramatically (1,2). This should be seen in the light of occasionally observed unexpected lack of local tumor control after seemingly inactivating radiation doses (3). The significance of cellular contact on radiation response is illustrated in the system of cellular agglomerates termed spheroids (4-7). It has been shown by Durand and co-workers (8), that Chinese hamster ovary (CHO) cells survive to a larger extent when irradiated in the form of spheroids comprised of 30-50 cells. Also, irradiating other cell types in spheroids with single doses increases survival (9,10). This state of enhanced resistance to irradiation persist for a period of time following dispersal of spheroids by trypsinization, and therefore could not be attributed to trivial physiological conditions such as hypoxia (8). Significantly, this state is not marked by changed population

composition, a factor which could alter radiation response in its own right. Inasmuch the cause of this phenomenon is not known, it should be noted that several factors may be involved in the response of spheroids to irradiation, some of which may be acting in opposite direction, as noted below. Apparently, epigenetic, in addition to genetic alterations are involved in radiation response.

The opposite effect in the modulation of radiation sensitivity under *in situ* conditions is also seen: tumor regression sometimes takes place after radiation doses which are not expected to have such an effect (11). Some of these manifestations could be apparently ascribed to the release of inhibitory growth factors, such as the Tumor Necrosis Factor (12), but more complicated processes may be also involved. One of these is the so called "Bystander Effect". The Bystander Effect (B.E.) is a chemo- and radiobiological phenomenon recently coming to the fore. While the mechanism of B.E. is poorly understood, it deals with the ability of cells affected by an agent to convey manifestations of damage to other cells, not directly targeted by the agent nor susceptible to it *per se*. In other words, in this phenomenon cells not able to process a chemical into a metabolite with chemotherapeutic action, acquire the ability to do so from other cells capable of such processing. Thus, the Bystander Effect is elicited by indirect means, by virtue of communication between cells which were directly affected by the agent, with those which were not. While the term "Bystander Effect" may be covering a variety of distinct mechanisms, the common denominator is that it relates to indirect

effects of (usually) low doses. The B.E. may have a pronounced role, as in protracted clinical treatment regimens, and in carcinogenesis.

Another area where cellular contact may be of paramount importance, is contact inhibition of proliferation. Here, normal and malignant cells respond differently, with normal cell proliferation being arrested upon contact between cells, while neoplastically transformed cells continue to proliferate after confluent growth has been achieved (13). This observation may be connected to the fact that cell-cycle progression is a prerequisite for 5-fluorouracil (5-FU) toxicity in cell culture (14), and the apparent differential 5-FU toxicity of neoplastic and untransformed cells (presumably because of their non-cycling status) (15). This in turn may conceivably account for the effectiveness of this drug in chemotherapy (16). We see this as an area to be exploited further, examining combined modality treatments in a system mimicking differential contact inhibition of normal and malignant cells.

All this points to the importance of a 3-dimensional cell contact in radiation and drug response such as may be found in tissues and solid tumors. The chief drawback to studying this effect in multicellular systems, was a lack of appropriate methods to measure clonogenicity. It should be recalled that clonogenic survival is the "gold standard" sought in tumor control, whereby not only temporary arrest, but permanent cure may be achieved. Consequently clinical trials were modeled after information gathered from systems where clonogenicity measurements were possible, i.e.,

single-cell monolayer cultures. Unfortunately, we now realize that relevancy of such studies to *in situ* conditions is lacking. Alleviation of this situation came with the introduction of our clonogenic Hybrid Spheroid Assay (HSA). In this system, test cells are surrounded by non-proliferating cells, where clonogenicity can be evaluated by simple statistical methods. This method is described elsewhere (17), but presently we want to explain how cell-cell interaction may be studied and what are the consequences of interrupting this interaction.

Consequences of agglomeration of live with dead cells.

When debating the possible extracellular contribution to radiation damage, there is a need to clearly delineate the site of the initial damage and its relation to clonogenic inactivation. Our own studies provide evidence that co-agglomeration of test with supralethally irradiated "feeder" cells in hybrid spheroids causes substantial sensitization of the former cells to radiation. It is the feeder cells which seem to exert a bystander effect, when in close contact with test cells. A requirement to elicit this effect, is that the hybrid spheroid aggregates must be dispersed by trypsinization (18). In essence, survival after repeated daily 2 Gy doses of x-rays, have an increasing lethal effect on HeLa test cells in (pseudo) hybrid spheroids with HeLa feeder cells. This effect is dramatically increased when AG1522 human fibroblasts in hybrid spheroids containing HeLa feeder cells, are irradiated with daily 1 Gy doses. Such treatment increases radiosensitivity (as

deduced from the survival curve parameter D_0) by a factor of almost two. Since a sensitizing effect is not elicited when cell agglomerates are not dispersed (19), it is evident that an element of physiological destabilization exerted by trypsin is involved. Furthermore, we observed that close contact of test and feeder cells is required for a maximum effect. Thus, the effect depends on close cellular contact, and not on the mere presence of a diffusible factor in medium. Together, our finding indicates that cell aggregation is both a stabilizer and a conveyor of potential fragility following irradiation.

The significance of our findings is twofold: first, we have shown that the radiation response is changing in the course of multifraction treatment, and second, that these changes can be exaggerated by additional treatment, such as trypsinization. How to translate the increased susceptibility to trypsin during the course of multifraction irradiation into therapeutic advantage, is an open question, but recourse to agents which are both potentially useful in chemotherapy, and affect intracellular structures also susceptible to trypsin remains a possibility. There are other advantages to our system, too, such as the ability to differentiate between contact inhibited and non-inhibited cells (because of the three-dimensional cell contact), equivalent to differentiating between normal and malignant cells. We therefore see the possibility of devising improved protocols using our system, and also of examining the innate response of tumor cells from surgical specimens. We anticipate therefore that we will be then able to

predict the outcome of a given procedure ahead of clinical trials.

Apart from what we are seeing as a potential improvement in clinical practice by using our system of hybrid spheroids, one may ask what is the significance of B.E.? The answer is that it introduces a new concept, which radically alters our view of how radiation interacts with the various manifestations of life. We are now coming full circle from an a) initial assumption of a global effects of irradiation (such as in the abscopal effect (20), to b) survival independence of individual cells (21), to c) multicellular interactive death (22). B.E. belongs to the last category, and it explains some poorly understood phenomena not discernible from conventional *in vitro* studies. Specifically, we are now in position to deal with the complexities of radiation response, and appreciate that processes leading to cell death are ongoing ones, which may be interfered with along the route. Thus the possibility must be considered, that radiation may act not only by targeting cells ultimately inactivated, but also through a larger volume than actually is the physical space of these cells (23). Moreover, with the incorporation of the B.E. concept in clinical radiotherapy research, some basic approaches to therapy have to be redefined.

Scope of the Bystander Phenomenon

The phenomenon of B.E. is currently under investigation in a number of laboratories. Historically, it evolved from the observation that susceptibility to a nucleotide analogue could be induced in animal cells by a viral gene (24). When this gene is transfected to cells

by viral vectors, cells with the functional gene produced the specific kinase for the conversion of the nontoxic prodrug to a highly toxic metabolite. This occurs because the fraudulent metabolite is incorporated into DNA, causing premature chain termination and cell death (25). The technique was originally hailed as an effective means of killing targeted tumor cells (26), but it soon became apparent that the phenomenon has broader implications. Namely, it was discovered that the toxic effect was by far too large to be accounted by targeted cells only; toxicity in a mixed cell population was extending beyond the initially transfected and therefore sensitive minority of the population, hence terming the phenomenon "Bystander Effect" (27). The opposite effect, i.e., the ability of healthy cells to rescue otherwise doomed cells (Good Samaritan Effect) was also described (28). In general, in its initial phase, studies on B.E., elaborated on interactions of cells transfected by viral vectors, with those which were not. The concept of the B.E. was soon enlarged, to encompass indirect mortality emanating from cells untransfected with viral vectors. These different situations are elaborated below.

Bystander Effect in Irradiated Cells

B.E. is also observed in cells exposed to ionizing radiation. Initially, the concept of B.E in radiobiology was associated with short-range, α particle irradiation. Here, the evidence relied chiefly on statistical considerations: with very small doses, one can obtain a situation where only a few of a multitude of cells is

traversed by an α particle. When after such doses, radiation effects are detected in a proportion of cells larger than expected, a case for the Bystander Effect is obtained. In a study by Little and associates (29), it was shown that the effect of α particles in the range of 0.03 to 0.25 cGy was propagated from target cells to bystander Chinese hamster ovary cells: it was noted that 30-50% of cells in the total population contained sister chromatid exchanges (SCE), when only less than 1% of cells were traversed by an α particle. A similar conclusion was reached by Deshpande and associates, also measuring SCE in human fibroblasts (30). In a more recent report by Little and associates (31), changes in the genetic make-up of an irradiated human fibroblast cell population was determined as a consequence of low doses of α irradiation (0.3-75 cGy). What was followed, was modulation of a number of cell-cycle regulatory proteins, formerly known as p53, p21, and p34, now termed TP53, CDKN1A and CDC2 respectively. Modulation of these gene products occurred in more cells than predicted purely from dosimetric and statistical considerations. Modulation was either by up-regulation (p53, p21), or down-regulation (p34), as revealed by Western analysis. Significantly, the altered cells are concentrated in clumps of neighboring cells, suggesting transmission of information from irradiated to non-irradiated cells. This effect can be abrogated by the application of the gap junction inhibitor lindane. Lindane (gamma isomer of hexachlorocyclohexane) has been shown to inhibit connexin, a major component of connexons in skin fibroblasts (32), which appears to increase in response to irradiation. The B.E. is not seen in non-confluent cultures of fibroblasts irradiated with low doses of α rays, signifying the

need for cellular contact for the expression of the phenomenon.

Bystander Effect and Carcinogenesis

The Bystander Effect is also demonstrated in other biological systems. It pertains to neoplastic transformation of contact-inhibited normal fibroblasts in culture. The procedure involves growing untransformed C3H10T1/2 mouse embryo fibroblast cells to confluence, when they become contact-inhibited, and then observing the appearance of supragrowing (on confluent monolayer) non-inhibited transformed cells, which form distinct colonies on the background of untransformed cells (33). When untransformed cells were exposed to short range β particles from Yttrium sources, cells in close juxtaposition to supralethally irradiated cells, became subject to the process of neoplastic transformation to an extent much larger (by a factor of 10) than cells not in contact with supralethally irradiated cells. Relevant to radiobiological phenomena, is the finding that destruction of gap junctions is instrumental in fostering carcinogenesis (34). Further development of adequate test systems to measure indirect carcinogenesis and survival levels after irradiation therefore assumes paramount importance.

Mechanism of Bystander Effect and of other manifestations of intercellular communication

When transmission of sensitivity to treatment with a certain agent

to neighboring cells was observed, the obvious question was asked what was the nature of this process? A major clarification came with the finding that B.E. could be abolished when direct contact between transduced and untransduced cells was prevented. This was achieved by the interposition of membranes (passable by the prodrug and its metabolites) (35). While direct contact between the two types of cells, transfected and untransfected, seems to be a requirement in this specific case, the direct cause of the B.E. response remains to be elucidated. The phenomenon may involve transmission of signals conveying damage, or it may even involve transport of material particles between the cells. Both apoptosis and phagocytosis seem to be involved (36-38), although it is not clear whether as a cause or as a consequence of B.E. Namely, bystander cell death can be increased by strategies which decrease or postpone apoptosis (39), and phagocytosis seemed to occur after apoptosis, indicating that their role in B.E. is indirect. Nor is octanol, an inhibitor of gap junction formation (40), capable of abolishing B.E. (but note that in radiological B.E. a gap-junction inhibitor had an effect - see above). This indicates that more than the establishment of intercellular contact is involved in the B.E. phenomenon, and that complicated feedback mechanisms and structural alterations in participating cells may be at work. Nevertheless, gap junctional intercellular communication is an important signaling event, and only after downregulation of such intercommunication can initiated cells proliferate and neoplastic transformation take place (34). Perhaps more informative is the observation that prevention of apoptotic vesicle transfer seems to diminish bystander killing (41). This points to the involvement of

some membrane material in intercellular communication. That membranes, albeit in a different context, may reconstruct into novel configuration, causing extensive functional perturbations, has been maintained by T. Alper (42). It is of interest therefore to contemplate the manner in which membranes may be involved in intercellular communication.

Conclusions

In conclusion, cellular interaction may be a multifaceted phenomenon, to include very diverse processes. It may lead to both increased survival after irradiation (as in the Good Samaritan Effect and in spheroids), and to decreased survival (as in the Bystander Effect). Furthermore, cell separation may both abrogate transmission of damage between cells, and destabilize nominally surviving clonogens. Nor has the disruption of gap junction a uniform response - it may prevent the Bystander Effect, or it may have no effect. Most likely, profound cellular changes are involved, presumably on the levels of (plasma) membranes, collateral to gap-junction formation. In spite of such complexity, cellular interaction is of much interest for our understanding of the process of carcinogenesis and of survival of clonogenicity after very small doses of an agent, especially radiation. Pending this understanding, we will be able to manipulate radiation response in a much more rational way and establish safer procedures in radio- and chemo-therapy.

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