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Intrinsic or Acquired Drug Resistance and Metastasis: Are They Linked Phenotypes?

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Abstract Evidence is reviewed which suggests a linkage may exist between certain forms of de novo or acquired drug resistance and metastasis. This includes the finding that expression of certain dominantly acting mutant oncogenes or tumor suppressor genes, i.e., genes which normally act to "drive" tumor progression and metastasis, can also affect the expression of drug resistance. Moreover, this can be accompanied by altered expression of certain cellular genes thought to be involved in expression of drug resistance. A direct linkage between acquired drug resistance and metastasis would suggest that tumor sublines selected for drug resistance should manifest more aggressive malignant properties than their drug-sensitive counterparts. While this does not appear to be true for drug resistant sublines selected in vitro, indeed such cell lines frequently manifest diminished in vivo tumorigenic and/or metastatic competence, there is some evidence to support such a correlation exists for tumor cell lines that are selected in vivo for drug resistance. Attention is also drawn to the fact that new linkages between metastasis and drug resistance may be uncovered by analyzing the ability of tumor subpopulations to acquire drug resistance after one or several previous exposures to chemotherapeutic drugs, as opposed to examining intrinsic drug resistance only. Furthermore, ability to detect induced or acquired drug resistance in vitro may be strongly influenced by the types of assay used to detect and monitor drug resistance. In particular, three-dimensional cell culture systems may reveal acquired or induced "multicellular" drug resistance in situations where conventional two-dimensional culture systems do not. Use of three-dimensional culture systems may therefore reveal as yet undiscovered associations between the phenotypes of metastasis and drug resistance.

Key words: tumor progression, oncogenes, multicellular tumor spheroids, drug resistance, metastasis

ON THE POSSIBLE RELATIONSHIP OF DRUG RESISTANCE AND METASTASIS

Two factors stand out for being responsible for the slow pace of progress made in developing more effective cancer treatments. These are, first, the ability of most types of cancer to metastasize, and second, their capacity to express resistance to anti-cancer therapies, including all major classes of drugs used in chemotherapy. Studies of metastasis and drug resistance have generally proceeded along separate pathways of research, but there are several reasons for thinking that a functional linkage between the two phenotypes might exist [Kerbel and MacDougall, 1992; Hennequin et al., 1993]. For example, there is a growing body of evidence (as will be summarized in a subsequent section of this paper) that expression of certain dominantly acting oncogenes or altered expression of tumor suppressor genes can enhance not only tumor cell growth and malignant aggressiveness, but also the relative expression of drug resistance [Hennequin et al., 1993]. Some of these genetic alterations, mutation of the suppressor gene *p53* being a good example, often occur in more advanced stages of disease [Lassam et al., 1993; Boyle et al., 1993; Mazars et al., 1991], and thus may be relevant not only to acquisition of malignant (i.e., metastatic) properties [Kemp et al., 1993], but also drug resistance properties as well. Initially, our reasons for investigating the possibility that a direct relationship between metastasis and some forms of drug resistance might exist arose as the result of prior studies on the "growth dominant" phenotype of metastatic cancer cells and the phe-

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nomenon of clonal dominance of primary tumors by metastatically competent tumor cell variants [Kerbel et al., 1988; Kerbel, 1990]. The latter refers to the ability of the metastatically competent tumors cells to gradually displace their non-metastatic cellular counterpart at the primary tumor site so that over time primary tumors may become phenotypically similar to distant metastases [Kerbel et al., 1988; Kerbel, 1990].

The metastatic cell clonal dominance phenomenon was discovered in our laboratory on the basis of a molecular genetic "tagging" strategy which allowed us to undertake complex clonal evolution studies of tumor progression in vivo [Kerbel et al., 1987; Waghorne et al., 1988; Korczak et al., 1988]. For example, metastatic variants of a non-metastatic mouse mammary tumor carrying a dominant selectable genetic marker (resistance to the antibiotic G418) were mixed together with the "unmarked" parental population. The marker is "inserted" by transfection of the tumors with a plasmid such as *pSV$_2$neo* or by infection with retrovirus vectors carrying the *neo* gene [Kerbel et al., 1989]. The random integration of the foreign plasmid or proviral DNA into the genome of a given clonogenic tumor cell can thus serve as a heritable genetic marker which can be detected by Southern blotting using the *neo* gene as a probe [Kerbel et al., 1989]. When mice were injected with mixtures containing an excess of non-metastatic tumor cells and a cryptic minority of tagged metastatic variants, the primary tumors at the site of inoculation became progressively overgrown by the progeny of the metastatic clone [Kerbel et al., 1987; Waghorne et al., 1988; Kerbel, 1990]. As reviewed elsewhere, there is evidence to suggest that a process akin to clonal dominance of primary tumors by metastatically-competent cells can take place during the growth of certain human tumors, including cutaneous malignant melanomas and colorectal carcinomas [Kerbel et al., 1988; Kerbel, 1990].

This type of clonal dominance process has several important implications, not the least of which is that it may clearly enhance the probability that primary tumors, even very small ones such as cutaneous melanomas, will generate sufficient numbers of potentially metastatic cells to allow formation of some distant metastases [Theodorescu et al., 1991]. We also speculated that the clonal dominance process could help explain the origins of some aspects of de novo or intrinsic multidrug resistance. The reasoning was as follows: it is now well known that the pattern of gene expression in metastatic cells can be very different from their non-metastatic precursors [Kerbel et al., 1988; Kerbel, 1990]. For example, a large family of diverse genes can be switched on in expression in metastatic cells, including genes encoding proteases, adhesion molecules growth factors, and motility factors, among others [Kerbel et al., 1988; Kerbel, 1990; Su et al., 1993]. Could some of these genes also include those which affect drug resistance? If so, then metastatically competent tumor cells would be expected, in general, to be more drug resistant than their benign cellular counterparts. Hence primary tumors could evolve to become increasingly drug resistant in the absence of drug exposure and selection, as the proportion of metastatically competent cells increased over time within primary tumors. Alternatively, it is also possible that highly metastatic tumor cell populations may be more adept at *developing* elevated drug resistance properties *after* exposure to chemotherapeutic drugs, i.e., they may be capable of an accelerated and/or elevated *acquired* (or induced) form of drug resistance even if they do not express differences in intrinsic drug resistance. The overall aim of this brief review is to summarize some recent evidence in support of the hypothesis that malignant tumor progression and acquisition of metastatic properties may indeed be associated with greater intrinsic levels of drug resistance and/or an elevated ability to acquire this phenotype after drug exposure.

EFFECTS OF ONCOGENES AND TUMOR SUPPRESSOR GENES ON THE DRUG RESISTANCE PROPERTIES OF TUMOR CELLS

The evidence is growing that most overt malignant cancers arise as the end result of an accumulation of genetic mutations involving a number of different genes [Fearon and Vogelstein, 1990]. Broadly speaking, these genes fall into two major operational categories: i) dominantly acting oncogenes associated with "gain-of-function" phenotypes (such as ability to produce mitogenic autocrine growth factors which stimulate cell division), and ii) recessive tumor suppressor genes, the inactivation and/or mutation of which is generally associated with "loss-of-function" phenotypes (such as loss of sensitivity to negative growth controls or loss of susceptibility to undergo apoptosis). Many of these genes encode transcriptional factors which can act to regulate the expression of other normal, cellular

genes [Fearon and Vogelstein, 1990]. Some of the cellular genes could conceivably affect the expression of drug resistance, and not just aspects of cell growth, differentiation, invasion, and metastasis. Indeed, as will be summarized below, various mutant oncogenes or tumor suppressor genes have been shown to affect the ability of tumor cells to intrinsically express drug resistance properties. Moreover, it is thought that highly malignant subclones of cancers have a greater total number of mutant oncogenes and tumor suppressor genes, in comparison to their benign counterparts [Fearon and Vogelstein, 1990]. Hence, the chances would be greater for a "genetic hit" to be present in highly malignant subclones which affect the phenotype of drug resistance. In addition, combinations of mutant oncogenes and tumor suppressor genes might augment the relative degree of drug resistance expressed (or the rate that it is acquired) just as they appear to enhance the degree of malignant aggressiveness expressed by tumor cell subclones [Fearon and Vogelstein, 1990].

There is, in fact, evidence to support the hypothesis that mutant oncogenes and/or tumor suppressors can augment drug resistance, based primarily on gene transfection studies. Table I summarizes the results of several such experiments. For example, transfection of *ras* or *myc* oncogenes into NIH 3T3 cells can result in increased intrinsic resistance to such drugs as cisplatinum, cyclophosphamide, adriamycin, and melphalan [Isonishi et al., 1991; Sklar, 1988; Niimi et al., 1991]. The results can be influenced by the oncogene or drug used, and the type of cell line used as the recipient. Thus, rat ovarian epithelial cells transfected with a mutant *ras* oncogene do not express an elevated intrinsic resistance to cisplatinum [Perez et al., 1993]. However, rat liver epithelial cells transformed with v-H-ras or v-raf oncogenes manifest an increase in resistance to various cytotoxic chemicals, including adriamycin and vinblastine [Burt et al., 1988], and moreover, this was accompanied by increased expression of the *mdr-1* and glutathione-S-transferase genes [Burt et al., 1988]. With respect to tumor suppressor genes, absence of the wild-type *p53* tumor suppressor gene renders cells more resistant to various DNA damaging chemotherapeutic drugs or gamma radiation, perhaps because of a failure to undergo drug-induced apoptosis [Lowe et al., 1993]. Similarly, tumors overexpressing the bcl-2 oncogene may also express an increased intrinsic resistance to various chemotherapeutic drugs [Reed, 1994; Fisher et al., 1993; Kamesaki et al., 1993; Walton et al., 1993]. This is because many chemotherapeutic drugs are thought to kill cells by inducing programmed cell death [see Eastman, 1990, for review] and bcl-2 acts as an inhibitor of programmed cell death [Reed, 1994].

TABLE I. Altered Drug Resistance Properties of Cell Lines Transfected With Dominantly-Acting Oncogenes

Oncogene	Recipient cell	Drug(s) tested	Observation	Reference
v-H-*ras* or v-*raf*	Rat liver epithelial cells	Adriamycin, vinblastine, 2-acetyl-amino-fluorene	Increased intrinsic resistance to all drugs	[Burt et al., 1988]
c-Ha-*ras*	NIH 3T3 mouse fibroblasts	Cisplatinum	8-fold increase in resistance	[Isonishi et al., 1991]
missense activated *ras* oncogenes	NIH 3T3 mouse fibroblasts	Cisplatinum	4.5–8.5 increase in resistance	[Sklar, 1988]
c-*myc* and/or c-H-*ras*	NIH 3T3 mouse fibroblasts	Cisplatinum, cyclophosphamide, adriamycin, melphalan	c-*myc* confers resistance to all drugs; *ras* to alkylating agents only	[Niimi et al., 1991]
c-*myc*	Mouse Friend erythroleukemia cells	Cisplatinum	Direct correlation of resistance with level of *myc* expression	[Sklar and Prochownick, 1991]
ras T24	Rat ovarian epithelial cells	Cisplatinum	No change	[Perez et al., 1993]
Bcl-2	Mouse FL5.12 prolymphoid progenitor cell line	Nitrogen mustard and camptothecin	2-fold increase in resistance	[Walton et al., 1993]

Thus, advanced cancers, which one might expect to harbor a number of genetic alterations such as overexpression of bcl-2 and inactivation/mutation of p53, should express, in relative terms, an "apoptosis-resistant" phenotype. This property would endow the tumor cells not only with an elevated ability to grow and survive in foreign tissue sites but to express (or develop) a drug resistant phenotype as well.

Various investigators have also assessed the effects of (mutant) oncogenes or tumor suppressor genes on the relative expression of unrelated genes (or their associated promoters) which are thought to be involved in acquisition of various forms of drug resistance. For example, two groups have independently obtained evidence that mutant *p53* genes can result in elevated expression of the *mdr-1* gene [Zastawny et al., 1993; Chin et al., 1992] whose product, P-glycoprotein, is thought to be involved in resistance to a variety of lipophilic natural compounds [Bradley et al., 1988]. Similarly, *ras* oncogenes may alter *mdr-1* gene expression [Burt et al., 1988; Hanania et al., 1991].

These findings are consistent with the notion that more advanced stages of tumor progression may be associated with an elevated ability of tumor cells to express drug resistance. We would also point out that the type of drug resistance investigated in the aforementioned studies was generally of the intrinsic (spontaneous) or de novo variety. It is possible that ability to induce or acquire resistance *after* drug exposure may be enhanced in advanced tumors (even if levels of de novo drug resistance are not). This is something which has rarely been investigated in the past.

ARE DRUG RESISTANT TUMOR SUBPOPULATIONS MORE MALIGNANT THAN THEIR DRUG-SENSITIVE COUNTERPARTS?

If the hypothesis is correct that growth-dominant ("progressed") metastatic cancer cells are "generically" more drug resistant, i.e., if the phenotypes are linked, then drug-resistant tumor cells might be expected to express a more aggressive malignant phenotype when compared to their drug-sensitive parental counterparts. However, there are several reports in the literature which indicate that if anything, cell lines selected in vitro for drug resistance usually manifest a non-tumorigenic phenotype in vivo or are strongly suppressed in their ability to form tumors and/or metastasize [Ganapathi et al., 1987; Giavazzi et al., 1983; Sircar et al., 1987; Kawai et al., 1990; Biedler et al., 1975; Belehradek et al., 1974]. Biedler has coined the term "reverse transformation" to describe the phenomenon of reduced malignant aggressiveness expressed by drug-resistant cells [Meyers et al., 1986]. Table II provides a summary of some of the literature published in this area. The basis for this curious phenomenon is unknown but could be explained by the mutagenic effects of prolonged exposure of tumor cells to very high concentrations of chemotherapeutic drugs in vitro. This could result, for example, in very high frequencies of immunogenic cell variants as has been shown to occur after exposure of tumor cells to alkylating agents or antimetabolites in vitro [Frost et al., 1984, 1987]. Whatever the mechanisms, these findings raise questions about the predominant use of cell lines selected in vitro for drug resistance after prolonged exposures to high concentrations of

TABLE II. Reduced Malignant Aggressiveness In Vivo Expressed by Drug Resistant Tumor Sublines Selected in Tissue Culture

Tumor	Drug	Observation	Reference
B16-BL6 mouse melanoma	Adriamycin	Decrease in lung metastases	[Ganapathi et al., 1987]
F4 rat brain tumor	MGBG (Methylglyoxal Bisguanlhydrazone)	Complete loss in tumorigenicity	[Sicar et al., 1987]
UV-2237 mouse fibrosarcoma	Adriamycin	Decrease in lung metastases	[Ginvazzi et al., 1998]
R1.1 mouse thymic lymphoma	Cisplatinum	Loss of tumorigenicity	[Kawai et al., 1990]
Chinese hamster ovary (CHO) cells	Vincristine or adriamycin	Loss of tumorigenicity	[Biedler et al., 1975]

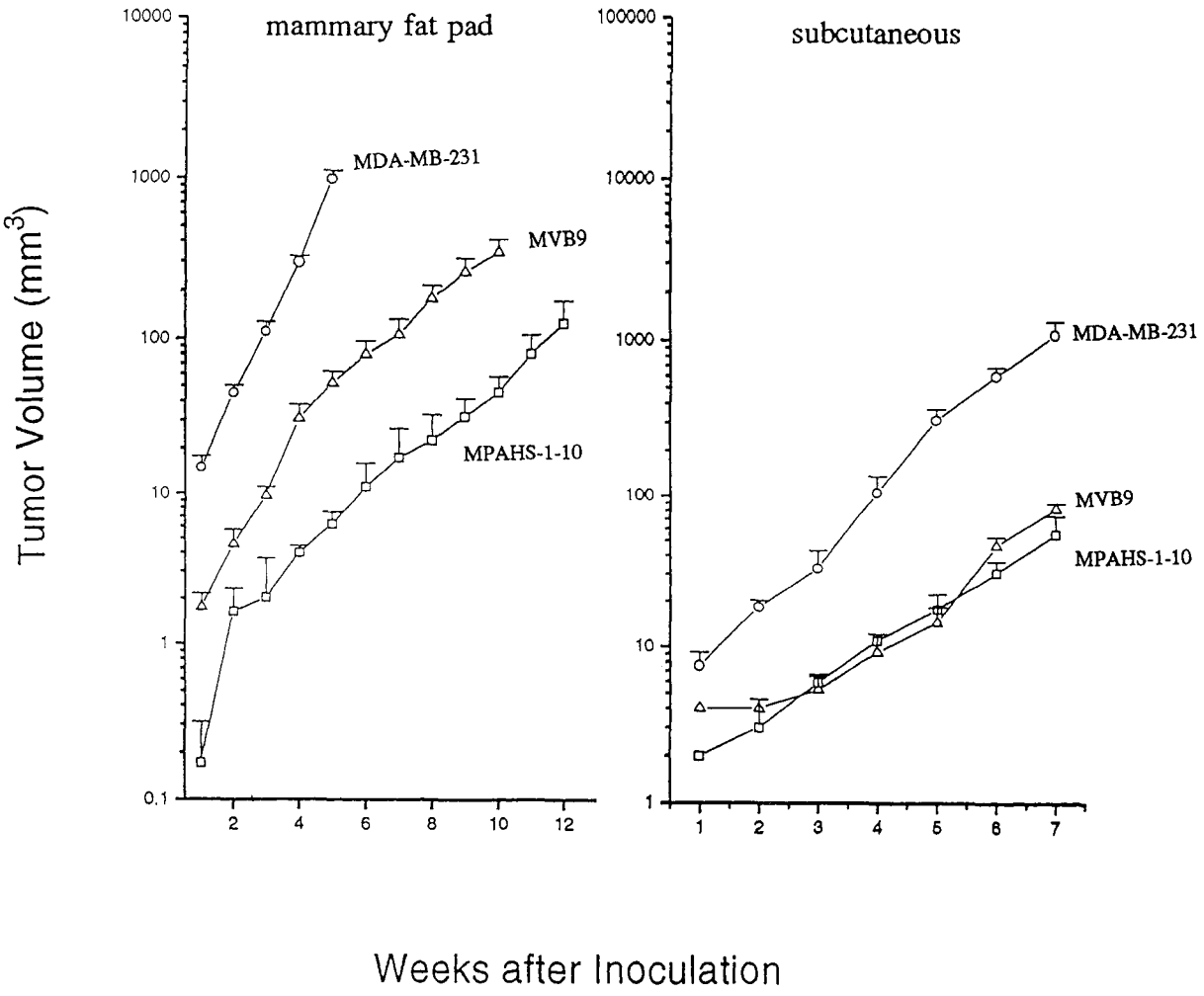

Fig. 1. Reduced tumorigenic properties of sublines of the human breast cancer cell line MDA-MB-231 previously selected for multidrug resistance in vitro. The MVB9 subline was selected by prolonged serial exposure to increasing concentrations of vinblastine in monolayer culture while the MPAHS-1-10 subline was obtained by transfection of MDA-MB-231 cells with a plasmid containing the mdr-1 gene. Both sublines expressed elevated levels of *mdr-1* and P-glycoprotein and expressed by a multidrug-resistant phenotype in vitro. The cell lines were kindly provided by Dr. Jeff Lemmontt. A total of 1×10^6 cells were injected into each of six female Swiss nude mice, either by the subcutaneous route or into the mammary fat pads, as indicated.

toxic drugs. This will be discussed in more detail in a subsequent section. An example, from our laboratory showing the suppression of tumor-forming ability (in nude mice) by drug resistant human breast cancer cells, is shown in Figure 1.

It is clearly possible, however, that an entirely different picture may emerge when drug resistant variants are selected in vivo. In this situation, there would be selection not only for the phenotype of drug resistance, but also for the most growth proficient subpopulations that exist among the surviving drug-resistant tumor cells. This latter, "host driven," selection would not generally exist in vitro where cells are generally grown under ideal, non-limiting tissue culture conditions. If this hypothesis is correct one might expect drug resistant subpopulations to be as tumorigenic, or more so, than their drug-sensitive counterparts in most cases. Similarly, their ability to metastasize should be unchanged or elevated. In fact there is evidence to suggest that experimental tumors selected for drug resistance in vivo are more malignant. A particularly interesting example of this comes from studies initiated in the laboratory of Teicher and colleagues [1990]. These investigators selected alkylating agent resistant sublines from a mouse mammary carcinoma called EMT-6 by serial passage in syngeneic Balb/c mice [Teicher et al., 1990]. After a 6 month selection process which

involved ten successive passages, the sublines selected were analyzed for drug resistance. High levels of resistance were expressed to the primary drug used for the selection, e.g., cyclophosphamide, cisplatin, thiotepa, or carboplatin, as well as lower levels of cross resistance to the unrelated alkylating agents [Teicher et al., 1990]. Of considerable interest was the finding that the drug resistant sublines failed to express their resistance properties in vitro in monolayer cell cultures: resistance was expressed only in vivo [Teicher et al., 1990]. A similar finding was noted by Starling [1990] who isolated sublines of a human lung adenocarcinoma for resistance to a vinca alkaloid-monoclonal antibody conjugate by serial exposure of tumor-bearing nude mice to the conjugate: the sublines expressed their resistance properties only in vivo.

In subsequent studies, Teicher and colleagues found that the EMT-6 primary tumor drug resistant sublines were much more proficient at forming spontaneous lung metastases after subcutaneous injection of the cells, in comparison to the drug-sensitive parental cell line [Teicher, 1993]. We have recently confirmed these findings by comparing the spontaneous metastatic properties of the various cell lines after orthotopic (i.e., intra-mammary fat pad) injection of the cell lines into female Balb/c mice (unpublished observations). The increase in metastatic aggressiveness in some cases was found to be quite striking, particularly with the cisplatinum resistant subline. Studies are in progress to evaluate the relative tumorigenic and metastatic properties of EMT-6 sublines selected for resistance in vitro to the same drugs by using "classic" long-term serial selection procedures. It would be of considerable interest to determine if such sublines express the "reverse transformation" phenotype, in contrast to the in vivo drug selected sublines.

Unfortunately, there are few reports in the literature describing the isolation of drug resistant sublines by in vivo selection procedures, followed by an evaluation of their relative growth properties in vivo. One of the few laboratories which has done so is that of Poupon and colleagues [Antoine et al., 1988]. These investigators compared the relative metastatic properties of chlorozotocin-resistant variants isolated in vivo from a rat rhabdomyosarcoma with those of their drug sensitive counterparts which had been serially passaged in vivo, without exposure of the animals to drug [Antoine et al., 1988]. A striking increase in ability to form spontaneous metastases in distant organs was expressed by the chlorozotocin-resistant selected tumor sublines when they were injected into mice without any drug treatment. The magnitude of the increase in metastasis was similar to that observed by Teicher [1993] when studying the alkylating-agent resistant sublines of the EMT-6 mouse mammary tumor, as discussed earlier [Teicher et al., 1990]. Poupon and her colleagues have also reported evidence that single chlorozotocin exposures of tumor-bearing animals can result in a similar "metastatic amplification" even if the growth of the primary tumor is suppressed [Pauwels-Vergely and Poupon, 1988; Poupon et al., 1984]. However, in these cases it is difficult to determine whether the enhancing effect on metastasis is due to drug-induced damage of host tissues, leading to elevated tumor cell arrest and/or growth at the sites of damage, or to a direct effect on tumor cell genotype and phenotype [McMillan and Hart, 1987]. In this regard it is important to note that there are reports showing pretreatment of animals with certain chemotherapeutic drugs can enhance the capacity of a subsequent injection of tumor cells to metastasize [reviewed in McMillan and Hart, 1987]. This drug-induced enhancement of metastasis can occur through a direct effect on tumor cell genotype and/or phenotype, as predicted in 1982 [Kerbel and Davies, 1982], as well as through an indirect process involving host tissues.

ACQUIRED "MULTICELLULAR" DRUG RESISTANCE AND ITS RELATIONSHIP TO METASTASIS

In the aforementioned studies of Teicher et al. [1990], mention was made of the fact that the EMT-6 sublines isolated in vivo for drug resistance did not express their resistance properties in vitro in monolayer tissue culture [Teicher et al., 1990]. When sublines grown in culture were returned to animals, the solid tumors which formed re-expressed their drug resistance [Teicher et al., 1990]. The results suggested the intriguing idea of a new mechanism of drug resistance operative "only in vivo" [Teicher et al., 1990]. In subsequent studies performed in our laboratory, we found that if the EMT-6 sublines were grown under more in vivo-like

conditions in tissue culture i.e., as three-dimensional multicellular aggregates (i.e., tumor "spheroids") they expressed their resistance properties in a manner strikingly similar to that observed in vivo [Kobayashi et al., 1993], as shown in Figure 2. This included levels of resistance and patterns of cross-resistance to other alkylating agents. Moreover, if the intact, drug-resistant spheroids were trypsinized, the single cells that were released from them largely failed to express their resistance when tested as monodispersed cell cultures [Kobayashi et al., 1993]. Taken together, the results suggested the existence of a new form of acquired drug resistance which is *not* (uni)cellular in nature, as are all other known or putative mechanisms of acquired drug resistance. Hence, we have designated this form of resistance as "acquired multicellular drug resistance" [Kobayashi et al., 1993].

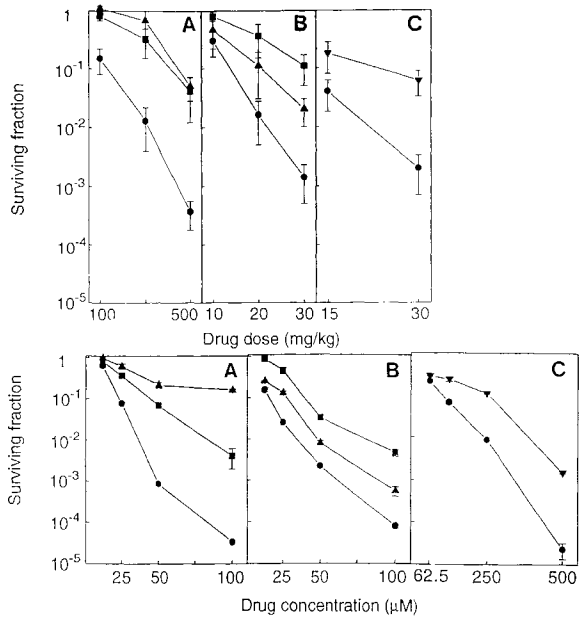

Fig. 2. Colony-forming assays representing either in vivo (top panel) or in vitro (bottom panel) resistance of EMT-6 cells selected in vivo for resistance to cyclophosphamide (CTX; ▲), cisplatin (CDDP; ■) or thiotepa (THIO; ▼), or control cells (●) passaged in vivo without drug treatment. Top panel: 24 h after i.p. injections of CTX, CDDP, or THIO (**A, B,** and **C,** respectively) into tumor-bearing mice, tumors were removed and single cell suspensions were prepared and plated for colony-forming assays. Bottom panel: colony-forming assay after 1 h drug exposure of each tumor line in three-dimensional culture. Following drug exposure, multicellular aggregates were dispersed into single cell suspensions which were then plated to determine colony-forming ability. All results are expressed as surviving fractions ± SEM of cells from treated groups, compared with untreated control groups. Adapted from Kobayashi et al. [1993], with permission of the publishers.

It may represent a form of group protection, providing "safety in numbers" for the majority of tumor cells populating a solid tumor mass. In this regard it is of interest to note that the morphology and structure of the multicellular aggregates was quite different between the drug-sensitive parental and drug-resistant sublines [Kobayashi et al., 1993]: the latter always formed much more compact, dense spheroids, in comparison to the parental population which formed large grape-like and loose clusters of cells [Kobayashi et al., 1993]. An example of this is shown in Figure 3.

In more recent studies, we have shown that multicellular drug resistance can be induced in EMT-6 mammary tumor cells by a single, transient exposure to agents such as cyclophosphamide or cisplatinum [Graham et al., 1994]. Moreover, the surviving cells could form highly compact multicellular spheroids [Graham et al., 1994]. In contrast, resistance was not observed (as expected) in conventional monolayer cell culture assay systems. These experiments were undertaken to address a particular paradox associated with the drug resistance literature, namely, why it usually can take very prolonged periods of time to isolate drug-resistant variants in tissue culture under ideal selection conditions, whereas drug resistance can apparently develop in an accelerated fashion in the clinical setting [Cadman, 1989]. This raises the spectre that some of the cellular resistance mechanisms uncovered in cell lines selected in vitro over prolonged periods of time may not be applicable to explaining the origins and mechanisms of drug resistance which develop in patients during or soon after chemotherapy [Cadman, 1989]. The sublines isolated by Teicher et al. [1990] took 6 months in mice, "equivalent," so to speak, to about 20 years in humans. We therefore decided to determine if drug resistance can be induced much more quickly than is generally thought to be the case by exposure and/or assay in three-dimensional culture systems. Our results indicate that this may indeed be the case [Graham et al., 1994]. An example of this is shown in Figure 4, in which mouse mammary EMT-6 tumor cells are tested for relative resistance to cyclophosphamide in monolayer versus three-dimensional culture conditions approximately 1 month after pre-exposure to the same drug. Resistance to cyclophosphamide was indeed induced but could be detected only by assay-

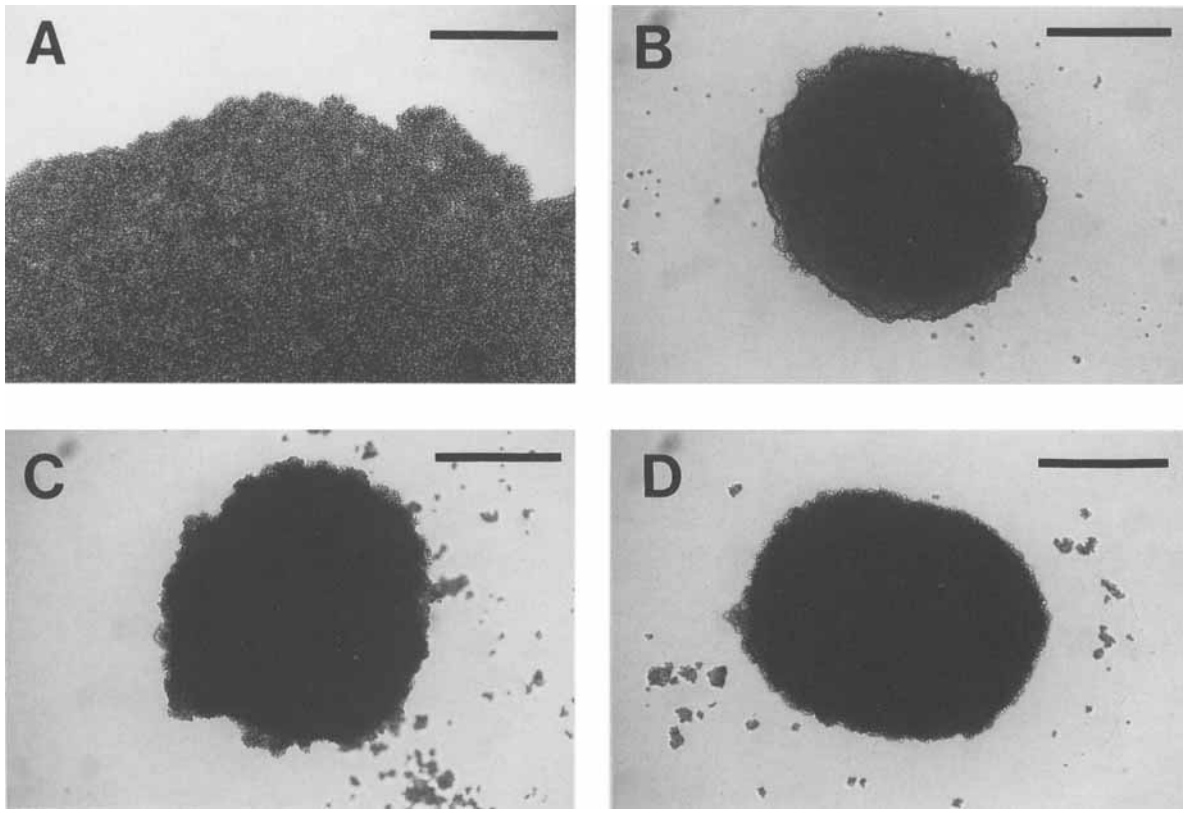

Fig. 3. Morphology of multicellular aggregates or tumor spheroids of EMT-6 mouse mammary tumor cells, and three drug-resistant sublines isolated from the EMT-6 tumor by serial exposure of Balb/c mice to various alkylating agents, as described by Teicher et al. [1990]. EMT-6/P (**A**) EMT-6/CTX (**B**), EMT-6/CDDP (**C**), and EMT-6/thio (**D**) refer to EMT-6 sublines selected for resistance to cyclophosphamide, cisplatinum, or thiotepa, respectively. Bars, 0.5 mm. The spheroids were grown by seeding 10^5 cells into individual 24-well tissue culture plates pre-coated with a thin layer of 1% agarose, as described by Kobayashi [1993]. Note the much more compact three-dimensional morphology of all of the drug resistant EMT-6 sublines in comparison to the drug sensitive EMT-6/P cell line. Adapted from Kobayashi [1993] with permission of the publishers.

ing for drug resistance using the three-dimensional culture assay system. This resistance was transient and was expressed only to the selecting agent [Graham et al., 1994].

IMPLICATIONS OF ACQUIRED/INDUCED MULTICELLULAR DRUG-RESISTANCE

The finding that drug resistance can be induced or acquired, albeit transiently, as a result of a brief exposure to a single drug, raises some important questions. For example, even if no differences in *intrinsic* drug resistance are uncovered when comparing highly metastatic tumor cell populations to their non-metastatic counterparts, it is possible that major differences in *acquiring* this phenotype may nonetheless exist. In this regard Hill and colleagues have reported that B16F10 melanoma cells have an enhanced ability to develop methotrexate resistance, due to gene amplification, in comparison to the B16F1 parental population [Cillo et al., 1989]. This involved multiple drug exposures over long periods of time. The possibility of detecting drug resistance after a single drug-exposure, by using three-dimensional culture assay systems, suggests that the issue of the nature of the interrelationship of drug resistance and metastasis should probably be re-evaluated in the context of the multicellular drug resistance assays and mechanisms. The same can be said for evaluating the effects of oncogenes and tumor suppressor genes on the expression of drug resistance. Not only should *intrinsic* drug resistance be examined, but also rapidly acquired or induced resistance, as well. We are therefore currently investigating the relative drug resistance properties of highly metastatic tumor sublines, in comparison to the parental non-metastatic cell lines

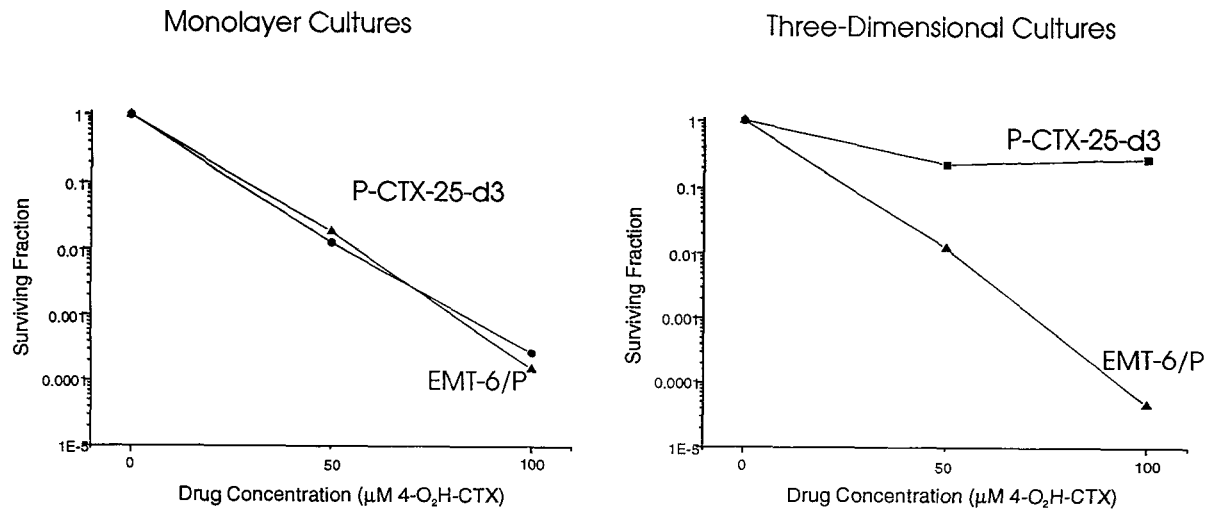

Fig. 4. Induction of drug resistance by a single previous exposure to a chemotherapeutic drug. The graph shows the survival of EMT-6 cells after 1 h in vitro exposure to 4-hydroperoxycyclophosphamide (4-O_2H-CTX). Cells were pre-exposed as three-dimensional cultures to a single dose of 4-O_2H-CTX (25 μM) and allowed to remain in such culture conditions for a further 3 days (■). Surviving cells were expanded as monolayer culture for 3–4 weeks before subsequent (i.e., secondary) drug exposure. Control cells (▲) were treated similarly but without any previous drug exposure. Symbols represent the mean surviving fraction ± S.E. of cells plated in triplicate wells. The resistance was not stable and disappeared within 3–4 subsequent population doublings. Transient drug exposure also resulted in an altered ability of the surviving cells to form multicellular tumor spheroids. Specifically the surviving drug exposed cells formed compact spheroids similar to that shown in Figure 3B,C,D. Adapted from Graham et al., in press.

from which they were derived, using conventional and multicellular drug resistance assays to examine both intrinsic and drug-induced (acquired) resistance.

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REFERENCES

Antoine E, Pauwels C, Verrelle P, Lascaux V, Poupon MF (1988): In vivo emergence of a highly metastatic tumour cell line from a rat rhabdomyosarcoma after treatment with an alkylating agent. Br J Cancer 57:469–474.

Belehradek Jr, J., Biedler JL, Thonier M, Barski G (1974): Actinomycin-D-resistant in vitro mouse cell line derived from a methylcholanthrene-induced sarcoma: Decrease of malignancy and antigenic characteristics. Int J Cancer 14:779–788.

Biedler JL, Riehm H, Peterson RHF, Spengler BA (1975): Membrane-mediated drug resistance and phenotypic reversion to normal growth behavior of Chinese hamster cells. J Natl Cancer Inst 55:671–680.

Boyle JO, Hakim J, Koch W, van der Riet P, Hruban RH, Roa RA, Correo R, Eby YJ, Ruppert JM, Sidransky D (1993): The incidence of *p53* mutations increases with progression of head and neck cancer. Cancer Res 53:4477–4480.

Bradley G, Juranaka PF, Ling V (1988): Mechanism of multidrug resistance. Bioch Biophys Acta 948:87–128.

Burt RK, Garfield S, Johnson K, Thorgeirsson SS (1988): Transformation of rat liver epithelial cells with v-Ha-*ras* or v-*raf* causes expression of MDR-1, gluthathione-S-transferase-P and increased resistance to sytotosic chemicals. Carcinogenesis 9:2329–2332.

Cadman EC (1989): The selective transfer of drug-resistant genes in malignant cells. In D. Kessel (ed). "Resistance to Antineoplastic Agents." Boca Raton, Florida: CRC Press, pp 168–183.

Chin K-V, Ueda K, Pastan I, Gottesman MM (1992): Modulation of activity of the promoter of the human MDR1 gene by ras and p53. Science 255:459–462.

Cillo C, Ling V, Hill RP (1989): Drug resistance in KHT fibrosarcoma cell lines with different metastatic ability. Int J Cancer 43:107–111.

Eastman A (1990): Activation of programmed cell death by anticancer agents: Cisplatin as a model system. Cancer Cells 2:275–280.

Fearon ER, Vogelstein B (1990): A genetic model for colorectal tumorigenesis. Cell 61:759–767.

Fisher TC, Milner AE, Gregory CD, Jackman AL, Aherne GW, Hartley JA, Dive C, Hickman JA (1993): *bel-2* modulation of apoptosis induced by anticancer drugs: Resistance to thymidylate stress is independent of classical resistance pathways. Cancer Res 53:3321–3326.

Frost P, Liteplo RG, Donaghue TP, Kerbel RS (1984): Selection of strongly immunogenic "tum" variants from tumors at high frequency using 5-azacytidine. J Exp Med 159:1491–1601.

Frost P, Kerbel RS, Hunt B, Man MS, Pathek S (1987): Selection of metastatic variants from a non-metastatic murine tumor after treatment with 2' deoxy-5-azacytidine or hydroxyurea: Implications for the mechanisms of tumor progression. Cancer Res 47:2690–2695.

Ganapathi R, Grabowski D, Schmidt H, Bell D, Melia M (1987): Characterization in vitro and in vivo of progressively adriamycin-resistant B16,BL6 mouse melanoma cells. Cancer Res 37:3464–3468.

Giavazzi R, Miller L, Hart IR (1983): Metastatic behavior of an adriamycin-resistant murine tumor. Cancer Res 43: 5081–5086.

Graham CH, Kobayashi H, Stankiewicz KS, Man S, Kapitain SJ, Kerbel RS (in press): Rapid acquisition of 'multicellular' drug resistance after a single transient exposure of mammary tumor cells to alkylating agents. J Natl Canc *Inst.*

Hanania N, Boyano M-D, Mangin C, Poupon MF (1991): Oncogene and MDR1 gene expression in rat rhabdomyosarcoma sublines of different metastatic potential. Anticancer Res 11:473–480.

Hennequin E, Delvincourt C, Pournay C, Jardilier J-C (1993): Expression of mdr1 gene in human breast primary tumors and metastases. Breast Cancer Res Treat 26:267–274.

Isonishi S, Hom DK, Thiebaut FB, Mann SC, Andrews PA, Basu A, Lazo JS, Eastman A, Howell SB (1991): Expression of the c-Ha-*ras* oncogene in mouse NIH 3T3 cells induces resistance to cisplatin. Cancer Res 51:5903–5909.

Kamesaki S, Kamesaki H, Jorgensen TJ, Tanizawa A, Pommier Y, Cossman J (1993): bel-2 protein inhibits etoposide-induced apoptosis through its effects on events subsequent to topoisomerase II-induced DNA strand breaks and their repair. Cancer Res 53:4251–4256.

Kawai K, Kamatani N, Georges E, Ling V (1990): Identification of a membrane glycoprotein overexpressed in murine lymphoma sublines resistant to *cis*-Diamminedichloroplatinum(II). J Biol Chem 265:13137–13142.

Kemp CJ, Donehower LA, Bradley A, Baimain A (1993): Reduction of p53 gene dosage does not increase initiation or promotion but enhances malignant progression of chemically induced skin tumors. Cell 74:813–822.

Kerbel RS, Waghorne C, Man SM, Elliott B, Breitman ML (1987): Alterations of the tumorigenic and metastatic properties of neoplastic cells is associated with the process of calcium phosphate-mediated DNA transfection. Proc Natl Acad Sci USA 84:1263–1267.

Kerbel RS, Waghorne C, Korczak B, Lagarde A, Breitman M (1988): Clonal dominance of primary tumors by metastatic cells: genetic analysis and biological implications. Cancer Surveys 7:597–629.

Kerbel RS, Cornil I, Korczak B (1989): New insights into the evolutionary growth of tumors revealed by Southern gel analysis of tumors genetically tagged with plasmid or proviral DNA insertions. J Cell Sci 94:381–387.

Kerbel RS (1990): Growth dominance of the metastatic cancer cell: Cellular and molecular aspects. Adv Cancer Res 55:87–132.

Kerbel RS, Davies AJS (1982): Facilitation of tumor progression by cancer therapy. Lancet ii:977–978.

Kerbel RS, MacDougall J (1992): Possible contribution of growth factors to the evolution of metastasis and de novo multidrug resistance in cancer. In B. Teicher (ed): "Drug Resistance in Oncology." New York: Academic Press, pp 583–602.

Kobayashi H, Man S, Kapitain SJ, Teicher BA, Kerbel RS (1993): Acquired multicellular-mediated resistance to alkylating agents in cancer. Proc Natl Acad Sci USA 90: 3294–3298.

Korczak B, Robson IB, Lamarche C, Bernstein A, Kerbel RS (1988): Genetic tagging of tumor cells with retrovirus vectors: clonal analysis of tumor growth and metastasis in vitro. Mol Cell Biol 8:3143–3149.

Lassam NJ, From L, Kahn HJ (1993): Overexpression of p53 is a late event in the development of malignant melanoma. Cancer Res 53:2235–2238.

Lowe SW, Ruley HE, Jacks T, Housman DE (1993): p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 74:957–967.

Mazars R, Pujol P, Maudelonde T, Jeanteur P, Theillet C (1991): *p53* mutations in ovarian cancer: a late event? Oncogene 6:1685–1690.

McMillan TJ, Hart IR (1987): Can cancer chemotherapy enhance the malignant behaviour of tumours? Cancer Metastasis Rev 6:503–520.

Meyers MB, Merluzzi VJ, Spengler BA, Biedler JL (1986): Epidermal growth factor is increased in multidrug-resistant Chinese hamster and mouse tumor cells. Proc Natl Acad Sci USA 83:5521–5525.

Niimi S, Nakagawa K, Yokota J, Tsunokawa Y, Nishio K, Terashima Y, Shibuya M, Terada M, Saijo N (1991): Resistance to anticancer drugs in NIH3T3 cells transfected with c-*myc* and/or c-H-*ras* genes. Br J Cancer 63:237–241.

Pauwels-Vergely C, Poupon M-F (1988): Chlorozotocin-induced selection of autocrine and multidrug resistant variants from a rat rhabdomyosarcoma. Anticancer Res 8:132–144.

Perez RP, Hamaguchi K, Tracey PA, Handel LM, Hamilton TC, Godwin AK (1993): Transformation of rat ovarian epithelial and rat-1 fibroblast cell lines by RAST24 does not influence cisplatin sensitivity. Cancer Res 53:3771–3775.

Poupon MF, Pauwels C, Jasmin C, Antoine E, Lascauz V, Rosa B (1984): Amplified pulmonary metastases of a rat rhabdomyosarcoma in response to nitrosourea treatment. Cancer Treat Rep 68:749–758.

Reed JC (1994): Bcl-2 and the regulation of programmed cell death. J Cell Biol 124:1–6.

Sircar S, Palkonyay L, Rodriques M, Allaire S, Horvath J, Thirion J-P, Weber J (1987): Isolation of variants resistant to methylglyoxal bis(guanylhydrazone) from adenovirus-transformed rat cells. Cancer Res 47:1339–1343.

Sklar MD (1988): Increased resistance to *cis*-diamminedichloroplatinum(II) in NIH 3T3 cells transformed by *ras* oncogenes. Cancer Res 48:793–497.

Starling JJ, Maciak RS, Hinson NA, Hoskins J, Laguzza BC, Gadski RA, Strnad J, Rittmann-Grauer L, DeHerdt SV, Bumol TF, Moore RE (1990): In vivo selection of human tumor cells resistant to monoclonal antibody-vinca alkaloid immunoconjugates. Cancer Res 50:7634–7640.

Su Z-Z, Austin VN, Zimmer SG, Fisher PB (1993): Defining the critical gene expression changes associated with expression and suppression of the tumorigenic and metastatic phenotype in Ha-*ras*-transformed cloned rat embryo fibroblast cells. Oncogene 8:1211–1219.

Teicher BA, Herman TS, Holden SA, Wang Y, Pfeffer MR, Crawford JW, Frei E, III (1990): Tumor resistance to alkylating agents conferred by mechanisms operative only in vivo. Science 247:1457–1461.

Teicher BA (1993): In vivo resistance to antitumor alkylating agents. In BA Teicher (ed): "Drug Resistance in Oncology." New York: Marcel Dekker, Inc., pp 263–290.

Theodorescu D, Cornil I, Sheehan C, Kerbel RS (1991): Dominance of metastatically competent cells in primary murine breast neoplasms is necessary for distant metastatic spread. Int J Cancer 47:118–123.

Waghorne C, Thomas M, Lagarde AE, Kerbel RS, Breitman ML (1988): Genetic evidence for progressive selection and overgrowth of primary tumors by metastatic cell subpopulations. Cancer Res 48:6109–6114.

Walton MI, Whysong D, O'Connor PM, Hockenbery D, Korsmeyer SJ, Kohn KW (1993): Constitutive expression of human *Bcl-2* modulates nitrogen mustard and camptothecin induced apoptosis. Cancer Res 53:1853–1861.

Zastawny RL, Salvino R, Chen J, Benchimol S, Ling V (1993): The core promoter region of the P-glycoprotein gene is sufficient to confer differential responsiveness to wild-type and mutant p53. Oncogene 8:1529–1536.