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Intrinsic resistance to anticancer agents in the murine pancreatic adenocarcinoma PANC02*

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Summary. PANC02 is a ductal adenocarcinoma of the pancreas that is resistant to every known class of clinically active antitumor agent. To study the mechanism(s) underlying the intrinsic drug resistance of this tumor, a mammary adenocarcinoma (CA-755) that also grows in C57/BL mice and is known to be drugsensitive was used for comparison. PANC02 resistance and CA-755 sensitivity to several antitumor agents and to X-ray therapy was confirmed in mice, and PANC02 also demonstrated relative resistance in tissue culture. Relative to Chinese hamster ovary (CHO) and CA-755 cells, PANC02 did not appear to show a higher rate of mutation to drug resistance in culture as based on the 6-thioguanine resistance marker. Although P-glycoprotein characteristic of the multidrug resistance (MDR) phenomenon could be demonstrated at the mRNA level using a sensitive RNAse protection assay, the level of expression found was several orders of magnitude lower than that observed in phenotypic MDR cell lines. Furthermore, quinidine failed to increase the sensitivity of PANC02 cells to Adriamycin under conditions that clearly potentiated the toxicity of the drug to a CHO cell line exhibiting classic MDR traits. The heterogeneity in the distribution of drugs was inferred as being significantly greater in PANC02 versus CA-755 cells in vivo as based on measurements of within-animal, within-tumor variance in the distribution of the marker compounds inulin and antipyrine. Although it may not be the only mechanism involved, this greater intratumor heterogeneity in drug distribution could theoretically play a major role in the intrinsic drug resistance of PANC02 in vivo.

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Introduction

The purpose of the present study was to determine the mechanism(s) underlying the intrinsic resistance to antitumor agents of a murine pancreatic ductal adenocarcinoma, PANC02, which was originally developed by Corbett and colleagues at the Southern Research Institute [4]. Certain features of PANC02 render it particularly attractive as a model to study new agents and to elucidate the bases of intrinsic as opposed to acquired drug resistance. For example, analogous to the corresponding human disease, it is refractory to all the major classes of antitumor agents, including nitrosoureas, anthracyclines, and alkylating agents, among others. Although other refractory tumors are known in mice, they respond to at least one class of antitumor agent; examples of such refractory tumors and drugs include nitrosoureas and alkylating agents vs B16 melanoma and Lewis lung carcinoma. Of 38 agents representing every known class of clinically active anticancer agent originally evaluated by Corbett et al. [4] for activity against PANC02, none achieved a gross $\log_{10}$ cell kill of >1.0 (tantamount to therapeutic failure) as judged by the criteria of these investigators.

Wilkoff and Dulmadge [19] originally reported the drug sensitivity of PANC02 in tissue culture; however, their retrospective comparison involved L1210 and P388 mouse leukemias and human neuroblastoma CHP-100 (summarized in [17]). Thus, some controversy regarding the in vitro resistance of PANC02 cells existed prior to the present study. We compared the in vivo and in vitro response to drugs of PANC02 with another adenocarcinoma that grows in C57/BL mice, namely, CA-755, which served as a drug-sensitive control. The underlying assumption of this work was that the mechanism responsible for the intrinsic resistance of PANC02 would be common for all currently available antitumor agents; that is, the occurrence of the number of mutations required to confer resistance to all anticancer drugs would seem to be highly unlikely. We established the in vivo and in vitro drug resistance and sensitivity of the two adenocarcinomas of interest and investigated certain parameters that might possibly account

Table 1. Therapeutic response of CA-755 and PANC02 to selected agents in vivo[a]

Treatment	Dose	Delay in tumor-volume doubling time (days)		Gross $\log_{10}$ cell kill	
		CA-755	PANC02	CA-755	PANC02
Adriamycin	10 mg/kg × 1	6.1	0.2	1.2	0.03
Cyclophosphamide	250 mg/kg × 1	> 7.5	1.6	>>1.5	0.2
6-Thioguanine	2 mg/kg daily × 5	> 7.5	3.0	>>1.5	0.4
Cisplatin	5 mg/kg × 1	1.5	2.0	0.3	0.3
X-ray	15 Gy	15.5	4.0	3.1	0.6

[a] CA-755 and PANC02 were implanted on opposite flank regions of C57BL mice on day 0. Drugs were given on days 9–12 when the tumor mass had reached approx. 500 mg. Tumor mass was estimated 3 times per week by caliper measurement, and $\log_{10}$ cell kill was estimated as described by Corbett et al. [4]. X-ray treatment was directed toward unilateral implants in the hind limbs

for the intrinsic drug resistance of PANC02. These include its propensity to mutate to drug resistance in vitro, the possible role of P-glycoprotein, and its intratumor heterogeneity in drug distribution. Greater intratumor heterogeneity in the distribution of inulin and antipyrine was observed in vivo in the PANC02 tumor as compared with CA-755. We propose that this feature of PANC02 provides a plausible explanation for its intrinsic drug resistance in vivo.

Materials and methods

Animal experiments. C57/BL male mice were used as host animals for PANC02 and CA-755 tumors. Tumor fragments (approx. 50 mg) were implanted s. c. by trocar into the flank region. In some cases, tumors were implanted bilaterally and on random sides, a procedure that did not alter the growth rate of either tumor. Drug treatments were begun when the tumors had reached a weight of $\geqslant 200$ mg, and tumor growth was determined by caliper measurement in two dimensions as previously described [15]. The method of Corbett et al. [4] for evaluation of drug response in terms of gross $\log_{10}$ cell kill was used. This procedure measures the growth delay due to drug treatment, and as reported by Corbett et al. [4], tumor regrowth rates determined following drug treatment were essentially identical to those observed in untreated animals.

For evaluation of the variance in the distribution of substances to the tumors, mice bearing bilateral implants were injected i. p. with 1 μCi [^{3}H]-inulin and 0.2 μCi [^{14}C]-antipyrine. Tumors were excised at 1, 2, 4, and 8 h thereafter. Each tumor was cut into at least five approximately equal fragments exhibiting similar gross features. Following solubilization in Protosol (New England Nuclear) and decolorization with hydrogen peroxide, radioactivity was measured by liquid scintillation spectrometry (Econofluor; New England Nuclear, Boston, Mass.) using a Beckman Model 7500 instrument. The comparison of within-animal, within-tumor analysis of variance was performed with repeated measures as described in the SPSS Command reference guide (MANOVA Command; SPSS Inc., Chicago, Ill.). To evaluate the response of these tumors to X-ray therapy, tumor fragments were implanted into the hind limbs instead of the flank region. The X-ray dose was delivered using the device previously described by Eifel [6], which was generously provided by Dr. L. Milas (Chairman, Department of Experimental Radiotherapy). Groups of five mice each were given doses of 10, 15, and 20 Gy. Tumor size was estimated by caliper measurement as described above.

Tissue-culture experiments. The PANC02 cells were generously provided by Dr. L. J. Wilkoff of the Southern Research Institute [19]. Wild-type CHO cells and a subline selected for resistance to Adriamycin that exhibited phenotypic MDR traits were provided by Dr. M. T. Kuo of this institution. A CA-755 cell line was established as described elsewhere [16]. Cells were treated continuously or for the times indicated in Results during exponential growth. Viability was assessed by colony formation as measured 7–8 days after the initiation of drug treatment as previously described [5]. X-ray treatment of cultured cells was performed analogous to the treatment of the hind limbs of mice by placing the tissue-culture dishes in the field of ionizing radiation.

P-glycoprotein mRNA determination. The mRNA for the mdr1 gene product P-glycoprotein was measured using a highly-sensitive RNAse protection assay as previously described [18, 20]. Briefly, a SPT19 plasmid containing the 211 nucleoticles (nt) Pvu II/KpnI fragment of mdr1 [20] was used to synthesize antisense RNA with [α-^{32}P]-uridine triphosphate (UTP). The probe was hybridized with total RNA that had been extracted using the guanidine isothiocyanate-cesium chloride gradient-centrifugation procedure described by Chirgwin et al. [3]. The hybridized complex was digested with ribonucleases, and the protected fragments were transferred to an 8% polyacrylamide-8 M urea gel and analyzed by autoradiography as described elsewhere [18].

Materials. [^{3}H]-Inulin (1.72 Ci/mmol) and [^{14}C]-antipyrine (45.5 mCi/mmol) were obtained from Amersham (Arlington Heights, Ill.) and New England Nuclear (Boston, Mass.), respectively. Adriamycin (Farmitalia Carlo Erba, Milan, Italy), cyclophosphamide (Asta-Werke AG, Bielefeld, FRG), and cisplatin (Bristol Laboratories, Syracuse, N. Y.) were obtained as injectable preparations from the University of Texas M. D. Anderson Hospital Pharmacy. 4-Hydroperoxycyclophosphamide was a gift from Dr. B. Andersson of this institution. Quinidine and 6-thioguanine were supplied by Sigma Chemical Co. (St. Louis, Mo.). Tissue-culture supplies were obtained from Gibco Laboratory (Grand Island, N. Y.).

Results

Since previous workers have compared the in vitro response of PANC02 with that of other histological types of mouse tumors and with that of human cell lines [19], we decided to use a mouse adenocarcinoma known to be drug-sensitive for such comparison. CA-755 is a murine mammary adenocarcinoma that is also syngeneic in the C57/BL mouse. Furthermore, many prior studies have demonstrated this tumor to be sensitive to a large number of anticancer agents. Table 1 demonstrates the sensitivity of this tumor to five different therapies and compares its response with that of PANC02 growing at a different site in the same treated hosts. For humane reasons, animals were not generally followed until their death; instead, tumors were allowed to regrow to a mass at least 3-fold that measured at the time of treatment, after which the mice were euthanized.

CA-755 was very responsive to most of the treatments shown, but, like the PANC02 tumor, it was relatively re-

Table 2. Cytotoxicity of selected agents to CA-755 and PANC02 cells in culture[a]

Treatment	ED_{50}		Relative ED_{50} (PANC02÷CA-755)
	CA-755	PANC02	
Adriamycin	0.021 μM	0.061 μM	2.9
4HC[b]	0.012 μM	0.024 μM	2.0
6-Thioguanine	0.02 μM	0.12 μM	6.0
Cisplatin	0.48 μM	1.13 μM	2.4
X-ray	4.2 Gy	10.1 Gy	2.4

[a] CA-755 and PANC02 cells were exposed to various concentrations of the drugs for 24 h. Viability of the treated cells was then determined according to colony formation [5]
[b] 4-Hydroperoxycyclophosphamide, an active form of the parent drug; cells were exposed to this agent for 1 h only
ED_{50}, Concentration required to reduce cell viability by 50%

Table 3. Rate of mutation to 6-thioguanine resistance in CHO, CA-755, and PANC02 cells[a]

Cell type	Mutation frequency
CHO	3.7×10^{-4}
PANC02	1.8×10^{-5}
CA-755	5.0×10^{-4}

[a] Mutants were selected in 10 mM 6-thioguanine as described by Huang et al. [13]

Table 4. Failure of quinidine to enhance the cytotoxicity of Adriamycin to PANC02 cells[a]

Cell line	ED_{50} (μM)	
	Quinidine	+ 10 μM Quinidine
CHO	0.038	0.031
CHO/MDR[b]	11.5	0.44
PANC02	0.035	0.035

[a] Cells in culture were exposed to various concentrations of Adriamycin in the presence or absence of 10 μM quinidine (the highest nontoxic concentration)
[b] Selected for overexpression of P-glycoprotein (>40 times) exhibiting all characteristics of the MDR phenotype
ED_{50}, Concentration required to reduce cell viability by 50%

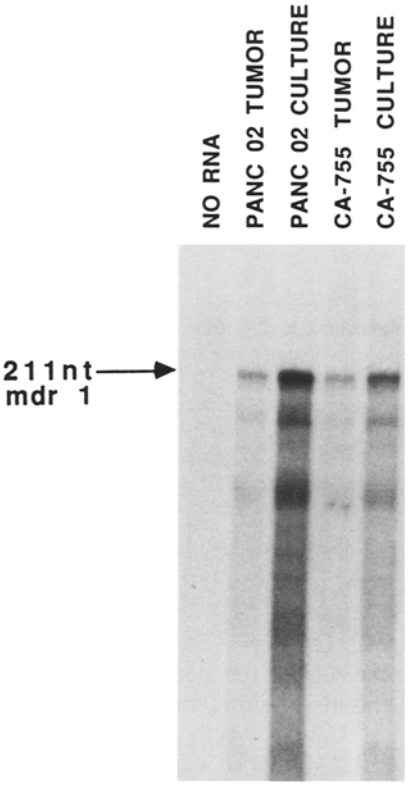

Fig. 1. Detection of mdr1 gene expression in mouse tumor and cell lines by ribonuclease protection assay. Total RNA (40 μg) was hybridized with 2×10^5 cpm ^{32}P-labeled antisense RNA probe and subjected to an RNAase protection protocol [18, 20]. The protected fragments were transferred to an 8% polyacrylamide-8 M urea gel and analyzed by autoradiography. *211 nt*, fragment specific for mdr 1

fractory to cisplatin. In confirmation of previous data regarding PANC02 therapy [4], none of the treatments elicited a response in excess of 1 $\log_{10}$ cell kill. PANC02 was also relatively resistant (2- to 6-fold) in tissue culture to the same treatments compared in vivo (Table 1 vs Table 2). This pattern of relative resistance in vitro has also been observed using toxic compounds not known to show anticancer activity, e. g., lipophilic organic cations and ouabain (data not shown). Thus, relative to CA-755, PANC02 appears to be resistant even when it is continuously exposed to toxic agents in vitro. Its relative resistance to X-ray therapy was approximately the same in vitro and in vivo (cf. Tables 1 and 2); however, the treatment was rather direct in each case, since tumors grown in hind limbs were directly exposed to radiation. As shown in Table 3, PANC02 cells did not appear to show a greater propensity to mutate to thioguanine resistance than did CHO or CA-755 cells. In fact, the mutation frequency at this locus was somewhat lower in PANC02 cells than in the other two cell lines investigated.

Using a sensitive RNAse protection assay [18, 20], we could readily detect mdr1 mRNA in PANC02 and CA-755 cells, regardless of whether they were derived from tumors in mice or from cells grown in tissue culture (Fig. 1). However, the level of expression in both cell lines was approximately equivalent to that observed in adult mouse kidney, and it would have been extremely difficult, if not impossible, to demonstrate using more classic approaches such as nick-translation-labeled cDNA in a Northern blot. Furthermore, the expression of message observed approximated that seen in wild-type CHO cells (data not shown). Further evidence that P-glycoprotein is unlikely to play a role in the intrinsic resistance of PANC02 cells is illustrated in the data presented in Table 4. Specifically, quinidine failed to potentiate the toxicity of Adriamycin to PANC02 cells in culture, whereas it did partially reverse the Adriamycin resistance of a CHO cell line demonstrating MDR features.

The within-animal, within-tumor heterogeneity in the distribution of two model compounds is summarized in Table 5. The distribution of inulin and antipyrine was significantly more heterogeneous (greater variance) in

Table 5. Intratumor heterogeneity in the distribution of inulin and antipyrine to PANC02 versus CA-755 tumors in C57/BL mice[a]

Radiolabeled compound	Within-tumor, within-animal ANOVA				
	Standard deviations (dpm/g wet tissue)				
	PANC02	CA-755	*F*	*df*	*P*
Inulin	6,022	4,298	1.96	67,65	<0.01
Antipyrine	1,089	675	2.60	67,65	<0.01

[a] Data represent standard deviations in the results of a multivariant analysis of variance (ANOVA) with repeated measures of distribution at 1, 2, 4, and 8 h after the i.p. administration of [^{3}H]-inulin and [^{14}C]-antipyrine to mice bearing bilateral implants. The average distributions at each time point were virtually identical, overall mean values being 33,682 and 35,930 dpm/g wet tissue for inulin and 3,349 and 3,194 dpm/g wet tissue for antipyrine in PANC02 and CA-755, respectively. Approximately 5 fragments obtained from 5 mice at each time point were analyzed

PANC02 as compared with CA-755. The ratio of antipyrine to inulin (a relative measure of the total water space to the extracellular space) was also significantly greater in the PANC02 tumor than in CA-755 cells (mean values, 0.111 ± 0.005 vs 0.098 ± 0.004; $n = 87$ vs 84; ratio of ^{14}C to ^{3}H expressed in dpm/g wet tissue; data not shown). This suggests that on average, the total water space is somewhat greater than the extracellular space in PANC02 tumors as compared with CA-755 tumors. The greater heterogeneity observed in antipyrine distribution can be more readily appreciated when the within-animal, within-tumor frequency distributions are plotted (Fig. 2).

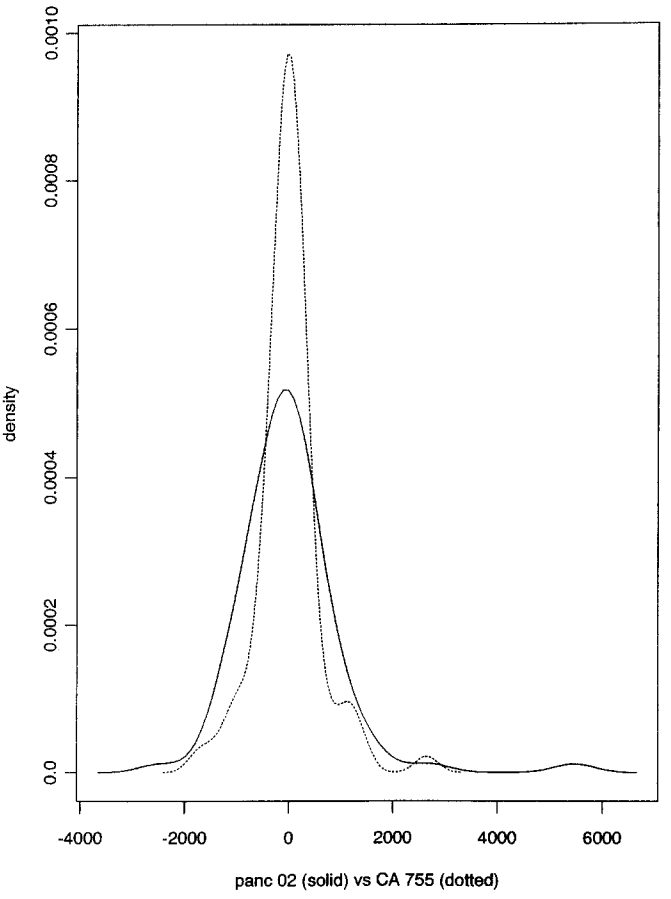

Fig. 2. Frequency of distribution for the uptake of [^{14}C]-antipyrine into CA-755 and PANC02 tumors in C57/BL mice. The intraanimal-intratumor distributions of radiolabeled antipyrine are plotted for the experiment described in Table 5 and in Materials and methods

Discussion

The phenomenon of "natural" or intrinsic drug resistance as it pertains to anticancer drugs has been appreciated for many years [1, 7, 10, 12]; however, the mechanisms underlying such resistance are not generally known. Much has been written and discussed regarding the possibility that intratumor heterogeneity in, e.g., drug distribution and drug-resistant mutations, might lead to treatment failure, but the relevance of such heterogeneity to therapeutic failure has for the most part not been conclusively demonstrated (reviewed by Heppner and Miller [11]). In the present study, we examined this and two other possible explanations for the in vivo refractoriness of PANC02 to anticancer drugs as summarized below.

In one approach, the frequency of mutation of PANC02 cells to thioguanine resistance in vitro did not appear to be greater than that of CA-755 or CHO cells (Table 3). Thus, a greater mutability for PANC02 as compared with CA-755 does not appear to be operative, at least when scored at this X-chromosome locus. Another approach tested the possible role of the MDR phenomenon involving overexpression of the membrane glycoprotein, P-glycoprotein [9]. Although the mRNA for P-glycoprotein could be demonstrated in PANC02 cells using a highly sensitive RNAse protection assay, this expression did not exceed that observed in the drug-sensitive reference tumor CA-755 (Fig. 1). Kessel and Corbett [14] have also reported low expression of P-glycoprotein in PANC02 cell membranes as assayed by Western blotting. Furthermore, quinidine failed to potentiate Adriamycin toxicity in these cells, although it did partially reverse Adriamycin resistance in a CHO cell line exhibiting the phenotypic features of MDR (Table 4). Analogous experiments using human renal adenocarcinomas led Fojo et al. [8] to ascribe the resistance of this human tumor to the MDR phenomenon. Due to these data and to the failure of PANC02 to respond to any class of antitumor agent, including antimetabolites and other drugs that are not affected by MDR, this phenomenon appears to be an unlikely explanation for the intrinsic resistance of PANC02.

In a third and more fruitful approach, we compared the in vivo distribution of two reference compounds, inulin and antipyrine, in mice bearing bilateral implants of CA-755 and PANC02. The intratumoral, within-animal heterogeneity in the distribution of both agents was significantly greater in the PANC02 cells as compared with the CA-755 tumor (Table 5, Fig. 2). Since these agents represent molecules that are either restricted to the extracellular space (inulin) or achieve distribution in the total body water (antipyrine), it seems reasonable to infer that the

heterogeneity in the distribution of many other drugs would also be greater in PANC02 than in CA-755. In fact, many anticancer drugs would show a distribution pattern similar to that of antipyrine. Thus, intratumor heterogeneity in drug distribution is an attractive hypothesis for the mechanism of intrinsic drug resistance in the PANC02 tumor in vivo. For example, given that a 1 $\log_{10}$ cell kill is the minimal requirement for a therapeutic response, the failure of only 10% of the tumor-cell population to be exposed to the drug concentration required to reduce cell viability by 90% (ED_{90}) would result in therapeutic failure. It would be interesting to evaluate regimens that might reduce this heterogeneity, perhaps by improving vascularity. A recent report indicates that metastatic PANC02 is curable by treatment with mitomycin C or porfiromycin in combination with interleukin-1α [2]. It is possible that the role of IL-1α in this effective drug combination would be to enhance the intratumor distribution of these agents. In this regard, as judged histologically, the PANC02 tumor did not seem to elicit an inflammatory response as marked as that evoked by CA-755 (data not shown).

PANC02 displayed reproducible resistance to agents in vitro as compared with CA-755, although the extent of such resistance was often small, being only 2–4 orders of magnitude for several agents (Table 2). Since it seems unlikely that heterogeneity in drug distribution would play a role in the resistance observed in the tissue-culture line, additional mechanisms may contribute to the refractoriness of PANC02. In another paper [16] we have presented evidence that the metabolism of 6-thioguanine differs in PANC02 as compared with CA-755 in a quantitative manner that probably contributes to the failure of PANC02 to respond to this antimetabolite.

One might suggest that PANC02 is simply more "normal-like" in its response to presently available antitumor agents; in other words, it lacks sensitivity to the mechanisms of action of such antiproliferative agents. It should be borne in mind that no given tumor is sensitive to all drugs in current use; a tumor will often respond well to only 3 or 4 of the 39 clinically available agents. In many cases the specific "sensitivities" are well explained. Thus, it is unlikely that heterogeneity is the only reason for the drug insensitivity seen in all tumors.

In summary, although it may not be the only mechanism involved, intratumor heterogeneity in drug distribution may play a major role in the in vivo intrinsic drug resistance of PANC02. The design of experiments to test whether this factor provides an adequate explanation for the resistance in this interesting experimental model is feasible.

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