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Perspectives and Commentaries

The Subrenal Capsule Assay: a Critical Commentary

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(A COMMENT ON: Mäenpää J, Kangas L, Crönroos M. Predictive testing of vulvar and cervical cancers to chemotherapy by the subrenal capsule assay. *Eur J Cancer Clin Oncol* 1985, **21**, 1141–1146.)

THE SUBRENAL capsule assay (srca) was proposed by Bogden *et al.* in 1978 as a rapid screening method for testing chemotherapeutic agents against human tumor xenografts [1]. Crucial technical aspects were: (1) the placement of 1 mm^3 fragments of tumor, a size small enough to allow initial nourishment by diffusion (2) placement under the renal capsule, a site chosen to provide a rich vascular bed and therefore good continued growth of the tumor fragments, as well as assurance of adequate drug delivery. Measurement, using an ocular micrometer, is made of the tumor fragment after trochar introduction under the renal capsule and again after the end of the assay period.

In their 1978 publication, nude athymic mice were used; treatment was given d1–10, and the final measurement done day 11. Well established tumor lines were used. In this paper, all control i.e. untreated tumors grew, and thus the issue of interpretation of control growth was not discussed. No statistical evaluation was done.

As a procedure to be used in nude, athymic mice, the srca was incorporated into the developmental therapeutics program of the NCI for a panel of human tumors to be used in the screening process.

The technique was extended to normal, immunocompetent mice in Bogden's second publication concerning the assay in 1979 [2]. The technique was similar in terms of tumor fragment preparation and placement under the renal capsule, but differed from the technique used for nude mice in that the time frame was reduced from 11 days to 6 days, with treatment given either daily days 1–5 or days 1, 3, 5. Having chosen the day 6 time point in order to avoid a primary immune response (usually originating on day 7) this paper concentrated on demonstrating adequate growth within this time period. Three human breast tumor lines were initially used, three colon carcinoma lines were also tested. Five of six of these tumors grew within the 6-day period, none doubled in size. All decreased in size when followed beyond 10 days; some tumor lines decreased in size as early as 6 days. Pretreatment with cyclophosphamide (150 mg/kg 24 hr prior to implantation) did not affect the growth of the MK-1 breast tumor, but did improve the growth of the colon line CX-1. For both tumors, the time over which tumors could grow before rejection in normal mice was increased by approx. 2 days. Finally, growth of both tumor lines and primary surgical explants were evaluated by implantation under the renal capsule of both nude athymic and normal immunocompetent mice. Of the four tumor lines tested, only one, the CX-1 Colon CA, appeared to grow better in the normal than the nude mouse host. More interesting is the data concerning primary human tumors, where 6/8 grew in normal immunocompetent mice in the subrenal location, as opposed to 2/8 transplanted into nude athymic mice. This data will become important relative to discussion later in this paper.

The 1981 paper of Boden *et al.* served to introduce the concept of using the srca as a prospective test of tumor sensitivity to chemotherapeutic agents [3]. Normal, immunocompetent mice were used and the 6-day assay performed. Tumor size was expressed as $(l + w)/2$ with TS defined as $(l + w)/2$ d6 $-(l + w)/2$ d0. In this paper, control *growth* i.e. a positive TS was required for the test to be evaluable. Fresh human tumor explants, primarily carcinoma of the breast were utilized. Re-

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sponse to therapy was defined as regression ≥ 15% of initial tumor size. Among breast tumors, their data demonstrated that for drugs tested, effectiveness occurred in the following order: cyclophosphamide > L-PAM > DES > Mtx > ADR > 5FU > Tamoxifen. This also needs to be considered according to later data.

Also of note, and something that cannot be overlooked given the climate of opinion that was present when this paper was published, is that these results, from the point of view of the number of tests which gave a result, (62%), were far better than what could be expected from the *in vitro*, clonogenic assay also being evaluated at this time [4, 5].

It is also at approximately this time, that I became involved in work concerning the srca being done at the Radiobiological Institute, in Rijswijk, the Netherlands. The work of Bogden had been followed there, and within the experimental chemotherapy division there was interest in its promise for both the screening of new drugs, and for patient related prospective tumor sensitivity testing. Having developed a familiarity with the technique, an initial review of our results led to the conclusion that certain technical aspects had not been completely analyzed.

Data was first presented in early 1983, demonstrating that homogeneity of the donor tumor was largely responsible for control growth within the time frame of the 6-day srca assay [6]. We also found that homogeneous tumor gave parallel growth when implanted subcutaneously in nude mice.

Having begun to evaluate the srca microscopically, we then began to evaluate the composition of the tumors which grew under the renal capsule in normal mice. Dr. H.H. Fiebig presented an abstract at the International Chemotherapy Congress in Florence in 1982, indicating that he was making the same analysis and had found there to be evidence of rejection of the human tumor explant in normal mice by day 6 [7]. He agreed to continue this work and we wrote a combined paper which appeared early in 1983 [8]. In this paper, treatment results of ovarian tumors, most of which were primary (1st transplant generation) with cyclophosphamide, adriamycin, 5-FU, and *cis*-platinum were compiled with the interesting result that by far the most responses were seen for cyclophosphamide. This occurred both using an arbitrary criterion, 40% reduction compared to control, and a Mann–Whitney-U test result with significance $P < 0.05$. A single response among 19 ovarian tumors was seen for *cis*-platinum, a result contrary to data concerning the clinical response rate to the drug. Another drug felt to be effective in ovarian carcinoma, adriamycin, not only produced only one response in 9/19 tumors treated, but tumors implanted under the renal capsule were larger than tumors implanted and not treated, (controls). When the histologic appearance of control and treated explants were examined by fixation and sectioning the kidneys after the final measurements had been done, an explanation was found for these results.

Tumors were found to be relatively variable in the amount of fibrosis and ingrowth of mouse host cells that occurred by day 6 of the assay, *and* the drugs tested had widely divergent effects on ingrowth of host cells. Cyclophosphamide, a drug widely used as an immunosuppressive agent, prevented ingrowth of host resistance cells completely in all but two tumors, while adriamycin and *cis*-platinum increased host responsiveness in most tumors tested. These data explained not only the failure of *cis*-platinum and adriamycin to produce macroscopic tumor reduction, but also the apparent increase in growth of adriamycin treated compared to control tumors. When two tumor early passage lines were tested both in normal and nude mice, one of the lines showed responsiveness to adriamycin, cyclophosphamide, and 5-FU in the nude but not normal mice, further when control tumors were measured, among these two ovarian tumors, three colon, and two lung tumors, in all cases but one they were larger in normal mice than nude mice, the difference attributed to host cells. These data also are important to the growth data in Bogden's 1979 paper previously mentioned.

At this point we felt that the technique might be improved if agents reported to be anti-inflammatory were used pre-implantation [9]. Cyclophosphamide, pre-irradiation (whole body), cortisone and silica were used; all proved useful except silica. Pre-implantation irradiation was adopted as our routine method, due to its simplicity, and experience was gained using 11 primary ovarian and 9 primary lung tumors. Despite pre-irradiation only 7/11 ovarian tumors and 3/9 lung tumors provided 6-day explants comprising more than 50% malignant tumor. For six early passage human tumors, a comparison was made between nude and normal hosts in growth and composition. For 4/6 tumors the degree of host infiltration and percentage tumor cells were virtually identical, leading us to suggest that part of the infiltration by non tumor cells was intrinsic to the 1 × 1 × 1 mm tumor fragment that was to be implanted. This was partially tumor origin dependent; on average ovarian tumors contained more 'tumor' per tumor than non-small cell lung tumors. Further, some tumors, being very homogeneous produce explantable fragments equally homogeneous; these produced tumor explants on day 6 which are composed exclusively of tumor cells in nude mice or

pretreated normal mice.

Limiting assays to these select tumors make the srca of little usefulness for prospective testing, but useful under the best conditions for screening.

An abstract presented at AACR in 1984 [10] from Bogden's group commented on the histologic appearance of explants, and concluded that 'pathologic quality control is necessary and feasible' but did not compare pre-treatment vs no pre-treatment of their normal mice.

Other investigators also found pre-treatment necessary or made other modifications to avoid the preventable host cell–cell infiltration that we had reported. Aamdal [13] used cell lines only and noted fewer problems than with primary tumors, and Levi [12] shortened the total assay time to 4 days in order to avoid a primary immune response. Most recently, Bennett *et al.* [11] confirmed both the need to pretreat, and host cell infiltration in the case of normal, immunocompetent mice in the absence of pre-treatment.

Griffin, Bogden, Reich *et al.* [14] published retrospective and prospective testing of the srca in a variety of tumors. A 38% response rate (14/37) was obtained by assay directed therapy, with some indication that the better the regression in the srca, the higher the likelihood that the patient would respond. They called, correctly, for more prospective studies.

In the interim, histologic studies were carried further, with multiple measurement and daily histologic sections being made for a panel of four human tumors implanted in pre-irradiated normal mice [15]. The impulse to study the kinetics of growth came from our prior histologic studies, which indicated that tumors had not completely 'settled' 24 hr after implantation, and that the process of adaptation to the subrenal capsule site might require more than the usual assay time frame. The results seen supported our initial findings, in that post-implantation there was a 2–3-day lag period before growth ensued in all four tumors. There were no day-to-day changes in tumor composition but by day 6 in all tumors, considerable decrease in tumor cell percentage with a concomitant increase in host cell infiltrate had occurred. Again, the variation in tumor fragments was such that the variation in fragments made from the primary tumor was frequently as great as the variation seen in tumor composition after 6 days of implantation even though day 0–4 tumor percentage was uniformly high in three of four tumors.

Finally, having decided that the only possible role for the srca might be found in screening of selected tumors, an analysis was made of the reliability of the srca in duplicate testing of tumors (early passage lines) against *cis*-platinum and three analogues [16]. *In-vitro* testing of the same tumors was done in parallel. For the srca, the greatest problem was lack of agreement between duplicated tests. Agreement was found in 35/60 tests, equal to the agreement expected if a random distribution were assumed. For the clonogenic assay, the major limitation was, as previously reported, the limited success rate, with only 21 of 40 tests producing at least 30 colonies. Reproducability in duplicate tests was adequate. For the srca, this would seem to form an important limitation even for screening; again limiting the tumor choice to those few both quite homogeneous and rapidly growing.

In light of this background, the paper of Mäenpää *et al.* [17] reporting on srca testing of vulvar and uterine cervical cancer is interesting. They performed the srca in its orthodox version i.e. without pre-treatment on normal mice. Drug combinations were generally tested, and only one sentence refers to possible histological 'quality control' — 'inflammatory reactions were found in the controls, the cytotoxic effect of a drug combination (adriamycin, cyclophosphamide, and *cis*-platinum) was clear ...'. That the combination contained cyclophosphamide, the only drug capable of producing effects in truly immunocompetent mice should raise no surprise given the previous discussion. Further, daily therapy was given without attention to possible anti-implantation effects. The three cases in which prospective confirmation of srca prediction could be evaluated proved less than convincing in that all had chemotherapy followed by surgery, in two after a single course, making evaluation of response less than complete.

Whether the srca has a future in screening of human tumors is as stated dependent on the selection of tumors with special characteristics making them suitable for the assay. Some human tumor lines, maintained in nude mice may meet the requirements. Further, it may be possible that some histologic types of cancer are more amenable to assay in the srca than others. It is, for example, possible that small cell tumors of the lung possess the set of characteristics required. In our experience, in the rare case that a large enough tumor was resected to make sufficient 'good' fragments, growth was good and explants were homogeneous. Technical improvements have been made; should all people still doing the assay wish to make further progress, a 'consensus' technique should be described and results; with proper histologic 'quality control' and verifiable standard for comparison (i.e. testing in nude mice) be complied in order to assign the technique to its place among sensitivity tests.

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