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ASSESSMENT OF TUMOR SENSITIVITY TO ANTICANCER AGENTS BASED ON TUMOR GRAFT VASCULARIZATION ON CHICK CHORIOALLANTOIC MEMBRANE (CAM)

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Summary

Tumors were implanted onto CAM where they were grown and the effects of anticancer drugs were examined following their injection into the yolk sac. To asses vascular response, the number of radially arranged capillaries was counted and compared with those in control CAMpreparations. By this method, tumor sensitivity toward selected anticancer agents could be determined more easily and at less expense than by other assay methods for estimating drng sensitivity. Neither does this method require mammals such as nude mice, elaborate animal room facilities or tissue culture systems.

Introduction

In the treatment of human tumors, a practical test to evaluate the relative efficacy of different anticancer drugs should be carried out at the start of clinical chemotherapy. Some authors suggested assays using cultured tissue[1], nude mice[2], and mouse subrenal capsule[3], but the effectiveness of such methods in chemosensitivity in clinical oncology has yet to be confirmed. Ingrowth of new capillaries, or angiogenesis, is required for tumor growth. Tumor grafts elicit centripetal angiogenesis with star-shaped of spoke-wheel capillary arrangements in CAM[4, 5]. Some methods have been developed to quantify vascular response in CAM and permit a more detailed examination of the sequence of capillary growth[5, 6]. In accordance with current animal welfare movements, this is a valid alternative assay system not requiring mammals.

Material and Methods

Tumor preparation:Murine mammary gland adenocarcinoma, canine transmissible veneral tumor (CTVT), rat fibrosarcoma and dog squamous epithelial cell carcinoma were used for assay. The tumors were maintained through successive generations by implantation of pieces of tumor into nude rats at our laboratory. All the tumors were transplantable and grew well on CAM.

The CAM - assay protocol:Several procedures for making a false air - space have been described. The procedure inroduced by Uchida et al.[7] was used in this study. At day 10 of incubation, the eggs were candled. Without damaging the shell - membrane, a hole was drilled into the shell where a false air - space had been produced by sucking air from the genuine air - space. A window of about 2.0 x 1.5 cm was opened at the site of this space. Solid tumor tissue samples were cut at 0.5 mm with a scalpel and placed directly on CAM. The windows were closed with aluminum sheets and sealed with tape and returned to the incubator. Three days after implantation, on day 13 of incubation, when vascularization of a successful graft was essentially complete, the aluminum covers were removed and the windows opened. Only eggs with normally developed embryos and blood vessel systems were used. Equal numbers of test and control eggs were randomly arranged

in pairs of 20 eggs. The drugs tested were Oncovin® (vincristine injectable, 0.1 mg/kg, Shionogi & Co., Ltd.) and Leunase® (L-asparaginase injectable, 0.1mg/kg, Kyowa Hakko Co., Ltd.). Since mean body weight of the chick embryos was 6.5 g, 0.065 mg of each drug dipssolved in 0.9 % NaCl solution was injected into the yolk sac. The windows were closed and The eggs placed the incubator. Four days later, on day 17 of incubation, CAMs were observed under a binocular microscope and removed from the embryo. They were extended, fixed with 10 % formalin, dehydrated and mounted onto glass slides with balsam. An equal number of control eggs received only 0.05 ml of 0.9 % NaCl solution.

Quantification of vascular response: Although a method using image analysis to quantify vascular response in CAM has been proposed by Jakob and Voss[5], the principes of our method are simpler and require no elaborate equipment. A thin transparent plastic sheet with a circle of 10 mm in diameter was placed on each slide in such a way that the central reaction focus of the CAM response was located at the center of the circle. Vascular organization was observed under a low-power microscope and the rate of vascular response assessed by counting vessels that had crossed the circle and reached the grafts. We defined a positive reaction as one that lost more than 30 % the radially arranged vessels since 1, tumors with vascular loss of more than 30 % showed extensive degeneation and/or necrotic lesions; 2, the fibroblastic organization exceeded half the grafts, and 3, cell division disappeared at this level of vascular response (Fig. 3-2). A negative reaction was defined as one showing a decrease of less than 30% the vessels and in which grafts showed mostly intact histological figures with cell division (Fig. 3-1).

The rate of vascular response in treated eggs was determined by dividing the number of spoke-wheel vessels by the mean number of those of control group. The average number of spoke-wheel vessels of control eggs was determined by dividing the total number of vessels by the total number of eggs. The number of eggs showing inhibited vascularity was counted and compared with that of control eggs. Since considerable variation in response within test and control groups was observed, X^2 test with 20 pairs in each experiment was conducted and significant individual results were obtained.

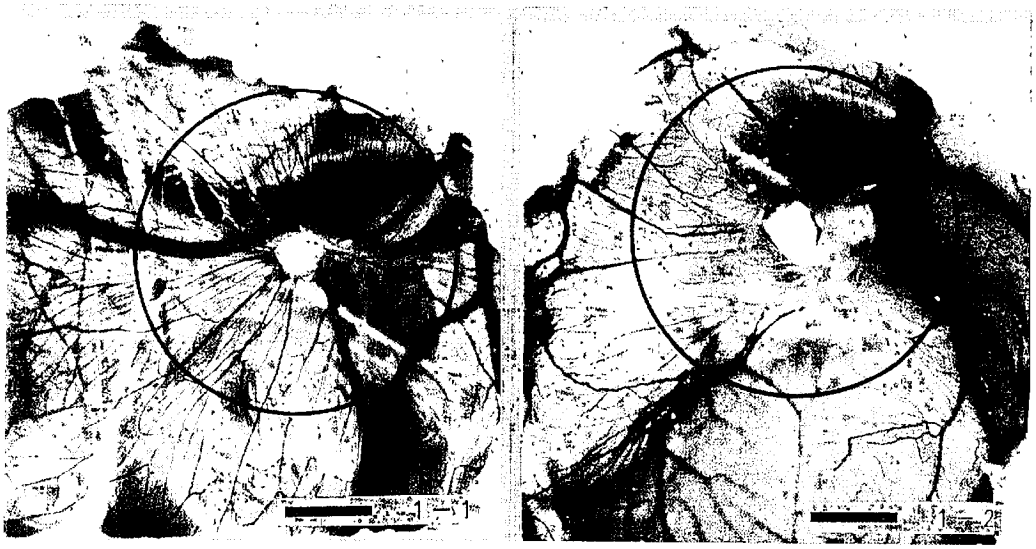

Fig. 1-1. Non-treated:Spoke-wheel capillary growth directed towards the tumor graft can be seen in a scanning circle on CAM. Murine mammary gland adenocarcinoma. Line = 20㎜
Fig. 1-2. Treated with anticancer drug (Oncovin):The spoke-wheel arrangement is lost and the number of capillaries within the circle is obviously reduced. Line = 20㎜

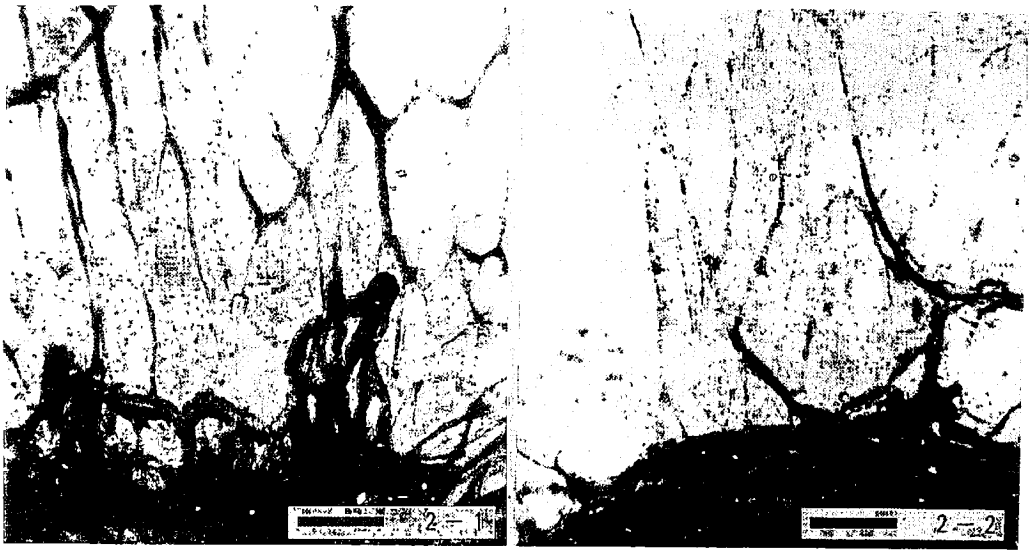

Fig. 2 - 1. Microphotograph of Fig. 1 - 1 showing a well developed capillary network around the graft. Capillary network in the graft is seen at bottom. The vascular system has been injected with Indian ink. x 40. Line = 100 μ m.
Fig. 2 - 2. Microphotograph of Fig. 1 - 2 showing shrinked capillary network. x 40. Line = 100 μ m

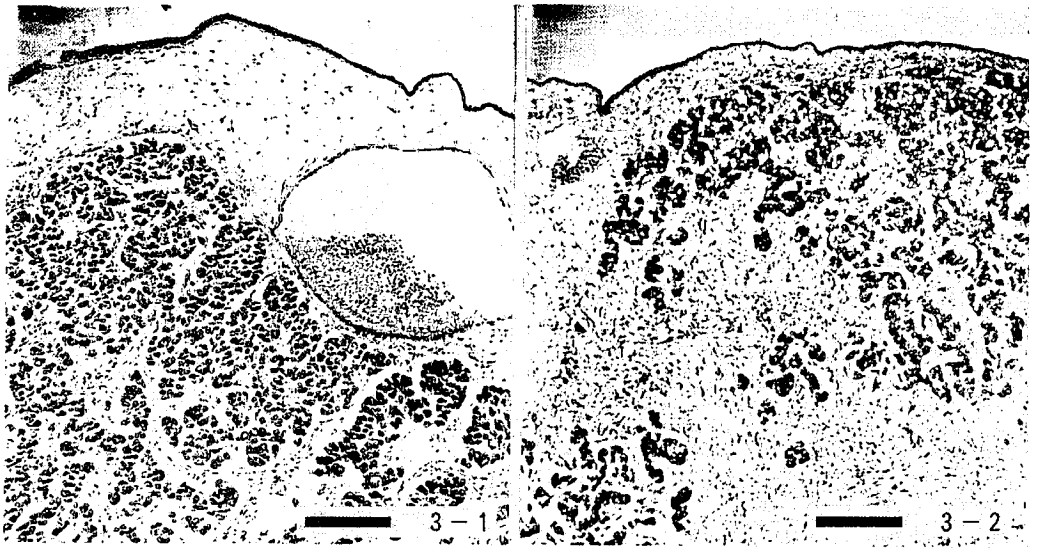

Fig. 3 - 1. Histological section of Fig. 1 - 1 showing well preserved tumor cells in the mesoderm of CAM. H & E. x 100. Line = 50 μ m
Fig. 3 - 2. Histological section of Fig. 1 - 2 showing extensive degeneration of carcinoma cells. H & E. x 100. Line = 50 μ m

Control eggs : Although one or two eggs in each group regressed in the grafts, most of the grafts showed good growth at 7 days following implantation. Surviving tumor tissue grew and spread beyond the original margin. Tumor growth was followed by some proliferation of small

Table 1 Assessment of sensitivity to anticancer agents by estimating vascularization on CAM in four tumors (number of positive/number of control eggs)

	squamous epithelial cell carcinoma	adenocarcinoma	CTVT	fibrosarcoma
Oncovin®	15/19	17/19	16/18	12/18
significance with X^2 test	$p<0.01$	$p<0.005$	$p<0.005$	$p<0.05$
Leunase®	12/18	16/18	11/18	12/19
Significance with X^2 test	$p<0.05$	$p<0.005$	$p<0.1$	$p<0.05$

vessels and increased centripetal vascularity with star-shaped or spoke-wheel formation (Fig. 1 - 1). All sprouts originating from the radially arranged vessels were observed to elongate and anastomose to from capillary networks (Fig. 2 - 1). Tumor cell proliferation was also notable in association with increased vascularity. The grafts were free from degenerating and/or necrotic materials (Fig. 3 - 1).

Anticancer agent - treated eggs : The grafts on CAM treated with drugs showed degenerative changes with pyknotic nuclei and vacuolatd cytoplasm deeply stained with eosin (Fig. 3 - 2). The degree of degeneration, however, varied according to the egg. The spoke-wheel arrangement disappeared and the numder of vessels was reduced drastically (Figs.1 - 2 & 2 - 2). The number of vessels provided valid indication of tumor sensitivity to anticancer drugs (Table 1).

Recently, Uchida et al.[7] introduced a chick-embryo chorioallantoic membrane (CAM) assay for determining the chemosensitivity of tumors. Their study involves measuring the volume of tumor grafts on CAM. Although promising in terms of predictive acuracy, it may be difficult to obtain reliable and reproducible results for tumor sensitivity to anticancer agents by measuring only tumor volume. The same volume of a tumor would not necessarily represent the same tumor growth activity, as the tumor may have degenerating and/or necrotic tissue in its neoplastic tissue. Therefore, there is the possibility that, when a tumor graft with degenerating tissue affected by an anticancer drug has the same volume as that of the untreated graft, the drung may be wrongly declared non - effective to a tumor. It is widely accepted that neovascularisation is an absolute requirement for tumor growth and vascular vessels are quite closely associated with tumor cell activity. Our new method presented here involving examination of tumor vascular characteristic may potentially overcome the disadvantage of the assay proposed by Uchida et al.. The test requires 7 days. This shorter period (7 days) may have obvious advantages in clinical situations. Permanent slides are made so that the results of the assay can be interpreted, rechecked, or compared to other assays at any convenient future time. Further work will be required to determine the accuracy and limitations of this assay.

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