were readily identified on the basis of exact matches of the energy spectra with those of the authentic compound spectra, and the major fragmentations observed are those expected (*9*). These data clearly demonstrate the capabilities of the MIKE technique for the direct analysis of plant materials.

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9. The most abundant ions in the cocaine spectrum are due to loss of 32 (16 percent), 106 (28 percent), 122 (100 percent), and 138 (12 percent). The corresponding ions in atropine are due to loss of 32 (100 percent), 94 (31 percent), 148 (30 percent), and 166 (56 percent).
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Chick Embryonic Skin as a Rapid Organ Culture Assay for Cellular Neoplasia

Abstract. *We used chick embryonic skin (CES) in organ culture to assess the neoplastic potential of a variety of cultured human and nonhuman cell lines. Cells derived from cancer tissues grew in CES and formed tumors in nude mice while cells derived from normal tissues grew in neither system. The CES proved to be more sensitive than the nude mouse when used to assay SV40 transformed human cells; each of four such lines grew in CES while only one of the four lines grew and formed tumors in nude mice. In addition, the patterns of invasion by inoculated cells can be easily studied in the CES. These results suggest that CES in organ culture offers an inexpensive, rapid, and reliable alternative to the nude mouse as a tumorigenicity test.*

Tumor formation at the site of inoculation has been the ultimate criterion for the transformed phenotype of tissue culture cells (*1*). For human cells in culture, tumorigenicity tests in vivo in xenogeneic hosts have been developed and include systems such as the nude mouse (*2*), the antithymocyte serum (ATS)-treated newborn hamster (*3*), the ATS-treated mouse (*4*), and the antithymocyte globulin (ATG)-treated nonhuman primate (*5*). While the formation of a tumor in these systems defines the transformed phenotype, the lack of tumor formation could be due to nonimmune rejection mechanisms (*6*), an insufficient number of cells inoculated (*7*), or other unknown factors (*8*). These models in vivo also have the disadvantages of maintenance and upkeep of animals, the need for immunosuppression, and occasionally lengthy times for tumor formation.

Because of these considerations and our preliminary findings that differences in sensitivity to tumor formation exist among various animal models, we were interested in developing a method for tumorigenicity testing that would be inexpensive, rapid, and reliable. Previous work suggested that the invasiveness of cells into chick embryonic skin (CES) might be related to malignancy (*9*). We found that human tumor cell lines did show extensive invasion in CES; however, we also found that normal fibroblasts such as WI-38 showed invasion as well. In attempts to find a marker for neoplasia other than invasion we considered the fact that tumor formation in xenogeneic models results from proliferation of inoculated tissue culture cells. We reasoned that CES in organ culture inoculated with cells would closely mimic the subcutaneous inoculation of cells in an animal, and that growth of the inoculated cells might then be considered equivalent to tumor formation in an animal. Further support for this idea came from early experiments in which the human tumor cell lines WiDr (*10*) and HT-1080 (*11*) formed macroscopic tumors in nude mice 2 weeks after inoculation, whereas the human diploid fibroblast line WI-38 (*12*) did not. Three days after inoculation we could find viable and actively mitotic WiDr and HT-1080 cells by microscopic examination of inoculation sites. In contrast, WI-38 cells were necrotic 1 day after inoculation with no mitoses and by 3 days no WI-38 cells were distinguishable. Thus, in vivo, the tumorigenicity of inoculated cells could be predicted by the presence of actively growing cells 3 days after inoculation.

Figure 1 illustrates the results when cells are inoculated onto CES. Figure 1A shows a fragment of CES inoculated only with medium after 3 days in culture. The skin was viable throughout its depth and showed the ability of the agar base to maintain tissue structure. Figure 1B shows a fragment of CES cultured for 3 days with WiDr cells. The skin substrate was viable and clusters of epithelioid cells with nuclei much larger than the chick tissue cells were seen. The cell clusters showed vigorous mitotic activity and invasion, and had the same histological appearance as tumors formed by the injection of WiDr cells into nude mice. The HT-1080 cells gave similar results. Another culture of CES with WiDr was minced and explanted into a 25-cm^2 flask (Costar). The resulting monolayer had a mixed pattern with many round colonies of large, epithelioid cells against a background of small fibroblasts. The epithelioid colonies had the same morphology as WiDr cells inoculated alone into parallel flasks. Poliovirus inoculated onto the mixed monolayer lysed only the epithelioid cells, indicating that they were of primate and not avian origin. As another test to demonstrate that the epithelioid cells were WiDr, the mixed monolayer was challenged with a medium containing only galactose as a sugar source, because chick cells cannot survive in such a medium (*13*). Most of the background fibroblasts died after 3 days while the epithelial colonies survived. In control experiments, chick cells derived from 9-day-old embryos cultured alone died in this medium while WiDr cells cultured alone survived. When WI-38 cells were cultured on CES for 3 days they showed no evidence of mitoses in the inoculated cells; in some cases the WI-38 cells became necrotic. Thus, the ability of inoculated cells to proliferate in the CES correlated with proliferation and tumorigenicity in vivo.

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We then evaluated the tumorigenic potential of a variety of cell lines in the nude mouse and compared the results to the ability of those lines to grow in CES as shown in Table 1. Cell lines derived from malignant tissues were, in general, tumorigenic in the nude mouse; the human neuroblastoma cell line IMR-32 proved to be tumorigenic in our hands only when inoculated intracerebrally. The neoplastic lines also showed extensive and consistent growth in the CES. Cell lines derived from normal tissues were uniformly nontumorigenic in the nude mouse, and, with one exception, showed no mitotic activity when inoculated onto CES. The exception was one WI-38 CES culture harvested 1 day after inoculation in which a single mitotic figure was found. The 29 cultures of WI-38 on CES held for 3 days or longer showed no mitotic activity even though viable cells could be seen. Thus, false positives appear to be eliminated by routinely culturing CES for a minimum of 3 days. The last group of cell lines was composed of cells originally derived from normal tissues and subsequently transformed in vitro by SV40 virus (*14*). Although human fibroblasts exposed to SV40 virus exhibit in vitro many characteristics of the transformed phenotype (*14*), their tumorigenicity in vivo has been debated (*7, 15*). We found that of four such lines only NVA-2 formed tumors in the nude mouse; however, all of these four lines showed abundant mitotic activity in the CES.

Other types of tumorigenicity assays in vitro have been proposed such as growth in soft agar (*16*), production of tumor angiogenesis factor (TAF) (*17*), and growth on contact-inhibited monolayers of mouse (*18*) and human (*19*) cells. However, these tests often pose inconsistencies when used to assay human cells. Normal human fibroblasts exposed to mutagens grow in soft agar but are nontumorigenic in nude mice (*20*), whereas a human bladder carcinoma cell line was tumorigenic in nude mice but did not grow in soft agar (*21*). HeLa cells are tumorigenic but do not always produce TAF (*22*). Finally, some human cell lines derived from bladder carcinomas form colonies on mouse cell monolayers but are nontumorigenic in nude mice (*21*), whereas some human fibrosarcoma and osteosarcoma cell lines are tumorigenic in ATS-treated mice but do not form colonies on normal human epithelial or fibroblastic monolayers (*23*). The inconsistencies cited may be related to the somewhat arbitrary conditions of tissue culture under which the cells are examined. For example, growth in soft agar can be enhanced or suppressed by varying the components in the agar (*24*); TAF production can be enhanced or decreased depending on the method of culturing the cells (*22*). There can be growth of cells on contact-inhibited 3T3 monolayers even though no visible colonies are formed (*25*); in addition, the normality of 3T3 cells has recently come into question since they can form vasoformative sarcomas in mice when implanted on glass beads (*26*).

In contrast, organ culture on agar maintains tissues in their normal architecture without the enormous proliferative and morphologic changes found when tissues are explanted into tissue culture (*27*). Thus, we have a system in which the ability of cultured cells to grow and form microscopic "tumors" in a normal tissue can be easily and rapidly examined. By isolating CES from the embryo we also eliminate any possible immunologic rejection mechanisms. Furthermore, the finding that in some cases viable WI-38 cells could be seen without mitoses suggests that lack of growth of inoculated cells is not an artifact of the method of inoculation as suggested for systems in vivo (*28*). We have shown the CES to be as reliable as the nude mouse in discriminating between human cells derived from cancer tissues and cells derived from normal tissues. The finding that only one of four SV40 transformed cell lines was tumorigenic in the nude

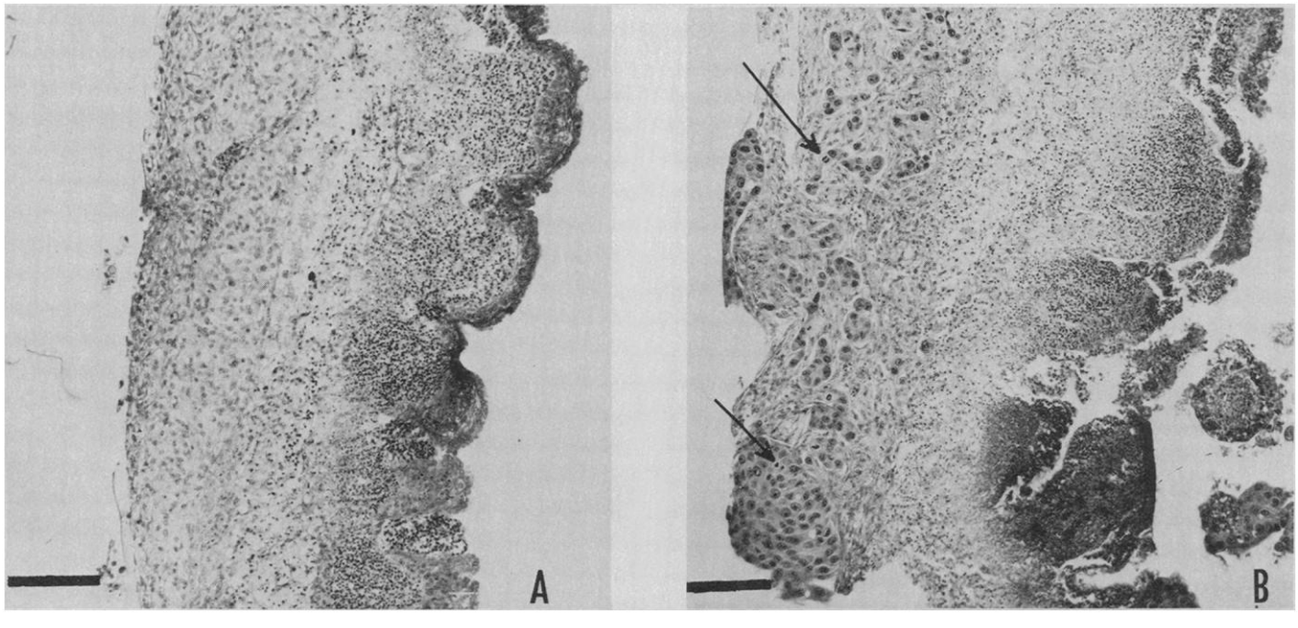

Fig. 1. Chick embryonic skin (CES) was obtained by blunt dissection of the dorsum of 9-day-old chick embryos (Truslow Farms) and placed feather side down on a modified Wolff agar base (*27*) composed of ten parts of 1 percent agar (Bacto-Agar) in Earle's balanced salt solution (EBSS) without bicarbonate, four parts fetal bovine serum (Reheis) and four parts chick embryo extract (prepared by extraction of minced 9-day chick embryos with EBSS overnight, followed by clarification by centrifugation at 2000*g*). WiDr cells (10^5) in 0.025 ml of Eagle's minimal essential medium (MEM) or MEM alone was dropped onto the free surface of the CES. After incubation at 37°C (in a humidified atmosphere for 3 days, the CES was fixed in Bouin's fluid and processed for paraffin embedding. Cut edges of CES were embedded on edge, and serial sections were cut and stained with hematoxylin and eosin. (A) Cross section of CES inoculated with MEM alone. (B) Cross section of CES inoculated with WiDr cells. The inoculated cells have distinctly larger nuclei than the chick cells; numerous mitotic figures (arrows) are visible. Scale bar = 0.1 mm.

mouse could be due to the fact that only 10^6 cells were injected. Koprowski and Croce (*7*) have shown that to form tumors in nude mice it may be necessary to inject up to 10^8 SV40 transformed human cells. Our findings that all four transformed lines showed vigorous growth in the CES with inoculums of 10^5 cells suggests that the CES may be more sensitive than the nude mouse in detecting the tumorigenic potential of SV40 transformed human cells. For these reasons we suggest the CES in organ culture to be a viable and inexpensive alternative to the nude mouse as a reliable tumorigenicity test for human cells in culture. It should be pointed out that this method does not evaluate such clinical findings of cancer as distant metastases; rather it establishes the ability of cells to form localized growths (microtumors) which could be benign or malignant. However, metastases from inoculated human cells rarely occur in tumorigenicity tests in vivo (*1–5*).

Because the evaluation is done by histopathologic examination rather than by gross observation for nodule formation, the biologic behavior of inoculated cells, such as patterns of invasion into a normal substrate, can also be examined. Invasion of tissues has been considered to be a marker of malignancy for cells (*9*); however, many normal types of cells can invade tissues in vivo. For example, fibroblasts will migrate through tissue to a wound site and lymphocytes can traverse endothelial walls (*29*). This probably explains our histologic findings of invasion seen with normal fibroblast cells. Thus, invasion as a general marker of malignancy for all cell types in culture has its shortcomings. However, normal epithelial cells do not invade tissues in vivo (*29*), which suggests that invasion may be a useful indicator of malignancy for cultured epithelial cells. Further study of this area is hampered at present by the paucity of well-characterized human epithelial cell lines. The vast majority of human cancers are carcinomas, that is, epithelial in nature. The CES may be a useful method to evaluate borderline lesions since invasive properties are easily studied in this assay.

Much of the present knowledge of cancer biology is based on experiments in which animal cells are used; however, there are many differences between animal and human cells in culture, and the extrapolation from animal tissue culture data to the human condition remains, at best, tenuous. For example, mouse fibroblasts in culture usually become aneuploid with unlimited lifespan (*30*), whereas many human fibroblasts remain diploid with a finite life-span (*12*). Although hamster cell cultures are easily transformed by chemicals (*31*) there have been few reports of chemical transformation of human cells derived from normal tissues (*20, 32*). Moreover, many of the nonhuman tumor cell lines contain endogenous viruses including retroviruses (*33*), while convincing evidence for a similar case in human tumor cells has yet to be established (*34*). Our findings that organ culture can detect the neoplastic potential of a wide variety of human cells derived from malignant tissues as well as those transformed by SV40 virus suggest that this assay may be of great value to basic human cancer research.

Table 1. Organ culture compared to the nude mouse as an indicator of neoplastic potential. Organ cultures were done as described in Fig. 1 and incubated for 2 to 8 days. At least 12 serial sections per culture were examined microscopically. A positive culture was defined as one in which mitotic figures could be seen in the cell inoculum. For the nude mouse test, 10^5 to 10^6 cells in 0.1 ml of Eagle's minimal essential medium was inoculated subcutaneously (sc); IMR-32 was also inoculated intracerebrally (ic). Macroscopic tumors that developed were excised, measured in three dimensions and confirmed by histopathologic examination. Mice were held to 56 days before declared negative. Source of cells: American Type Culture Collection: CCL-54, CCL-75.1, CCL-95.1, IMR-32, HT-1080; R. Wallace, Lederle: WiDr, LED-T_1; Experimental Biology Branch, Bureau of Biologics: HeLa, FRhL-2, MJ; C. Croce, Wistar Institute: LNSV, NVA-2; Cell Biology Branch, BoB: WI-38.

Origin	Species	Cell name	Reference	Organ culture (number positive and number inoculated)	Nude mouse (number of tumors and number of inoculations)
		Neoplastic cells			
Colon adenocarcinoma	Human	WiDr	(*10*)	62/67	52/54
Cervical carcinoma	Human	HeLa	(*35*)	32/35	13/20
Cervical carcinoma	Human	LED-T_1 (HeLa)*	(*36*)	25/28	8/8
Neuroblastoma	Human	IMR-32	(*37*)	12/12	0/4 sc; 3/3 ic
Fibrosarcoma	Human	HT-1080	(*11*)	17/19	10/10
		SV40 virus transformed fibroblasts			
Embryonic lung	Human	CCL-75.1†	(*14*)	13/13	0/5
Embryonic lung	Human	CCL-95.1‡	(*14*)	7/7	0/5
Skin	Human	LNSV	(*14*)	1/3	0/4
Skin	Human	NVA-2	(*14*)	2/3	5/9
		Normal (nonneoplastic)			
Embryonic lung	Human	WI-38	(*12*)	0/29	0/20
Skin (Trisomy 21)	Human	CCL-54	(*38*)	0/8	0/9
Embryonic lung	Rhesus	FRhL-2	(*39*)	0/9	0/5
Skin	Muntjac	MJ	(*40*)	0/16	0/5

*Although originally described as an SV40 transformed human fibroblast cell line, LED-T_1 was found to have four HeLa marker chromosomes and type A glucose-6-phosphate dehydrogenase isozyme. †Derived from WI-38. ‡Derived from WI-26.

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Relative Risks of Saccharin and Calorie Ingestion

Abstract. *The risk of a person getting cancer from ingesting saccharin is compared with the risk of ingesting additional calories which cause excess body weight. It is found that, for a person who is 10 percent overweight, the risk of ingesting one diet soft drink, which would cause a decrease in life expectancy of 9 seconds, is approximately equal to the risk of ingesting one additional kilocalorie; that is, if ingesting a diet drink inhibits ingestion of more than 1 kilocalorie, its benefits exceed its risks.*

The reason for the use of saccharin is to avert the ingestion of calories. Therefore, to make a risk-benefit analysis of this process one must know the relative risks of saccharin and calorie intake. It is the purpose of this report to develop a comparison between the two.

In the recent Canadian study of patients with bladder cancer (*1*), a link was established between that disease and use of saccharin such that if the U.S. population (2×10^8) were to ingest one 12-ounce diet soft drink (*2*) per day throughout their lives, there would be an extra 1200 bladder cancers per year. This implies a risk of 1200 cancers per 7.3×10^{10} drinks, or one cancer per 6×10^7 drinks. There is ordinarily a time delay of 10 to 50 years between ingestion of a carcinogen and development of a cancer, so an average case would result in no more than a 20-year loss of life expectancy; thus an average diet drink would reduce life expectancy by 20 years per 6×10^7, or about 9 seconds. To put this number into perspective, let us consider that smoking a single cigarette reduces life expectancy by 12 minutes (*3*), so a diet soft drink is about 80 times less dangerous than a cigarette. From the above result (or from the original finding) it is straightforward to calculate that one diet drink per day throughout life causes a reduction in life expectancy, ΔL, of 2 days; or

$$\Delta L = 2 \text{ days} \left(\frac{\text{diet drinks}}{\text{day}} \right) \quad (1)$$

The benefits of diet soft drinks result from their use in weight control by reducing caloric intake. Being overweight is well known to reduce life expectancy. In a somewhat earlier study, Pauling (*4*) analyzed the data in a 1952 report (*5*) to obtain best fits to both linear and quadratic relations between loss of life expectancy, L, and overweight, $(W - W_0)$, where W is the weight and W_0 is the optimal weight. These were

$$L = 17 \text{ years } [(W - W_0)/W_0] \quad (2)$$

$$L = 36 \text{ years } [(W - W_0)/W_0]^2 \quad (3)$$

If one differentiates Eq. 3 and assumes that an average saccharin user is at least 10 percent overweight and weighs perhaps 160 pounds (73 kg), one obtains

$$\Delta L = (0.05 \text{ year/pound}) \Delta W \quad (4)$$

If one applies the same assumptions to Eq. 2 one obtains

$$\Delta L = (0.11 \text{ year/pound}) \Delta W \quad (5)$$

independent of the percentage of overweight. There are more recent data on this subject (*6*). To quote typical figures, for a 45-year-old male with an optimal weight of 150 pounds, an increase in weight to 170 pounds reduces his life expectancy by 1.5 years; an increase to 200 pounds reduces it by 4 years. This gives an approximately linear relation with

$$\Delta L = (0.08 \text{ year/pound}) \Delta W = (29 \text{ day/pound}) \Delta W \quad (6)$$

Since this is intermediate between Eqs. 4 and 5 and is based on better data, I use Eq. 6.

An average person's body weight is related to his average daily caloric intake at about 1 pound per 14 kcal/day (*7*). If one multiplies this by Eq. 6 one obtains a change in life expectancy

$$\Delta L = 2 \text{ day/kcal-per-day-intake} \quad (7)$$

By comparing Eqs. 1 and 7 one can see that diet soft drinks give a net benefit if one such drink reduces caloric intake by more than 1 kcal.

There seems to be no firm evidence on the amount by which diet drinks reduce caloric intake (or body weight). A nondiet drink contains about 100 kcal, so if all other things were unchanged, the substitution of diet for nondiet drinks would increase life expectancy by 100 times more than the cancer risk reduced it. This is perhaps an extreme assumption, but it seems most unlikely and it would be very difficult to prove that it overestimates the reduction in caloric intake from diet drinks by a factor of 100. Unless this is done, there is no evidence that the risk of diet drinks is greater than their benefits.

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