the hypothesis that AL are the common kind of precancerous lesions in the human breast. In our opinion, every effort should be made to prove or disprove this hypothesis by direct experimental means.

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Messenger RNA Induction of Fast Sodium Ion Channels in Cultured Cardiac Myoblasts

Abstract. *Incubation with adult heart messenger RNA caused the appearance of fast sodium ion channels in young myocardial cells whose development had been arrested in vitro. The induction was blocked by cycloheximide, indicating dependence on protein synthesis. Thus, cardiac myoblasts can be made to differentiate in vitro, and membrane properties can be altered by exogenous RNA.*

Electrical activity can be recorded in the precardiac areas (anterolateral blastoderm) of the 20-hour embryo (*1*) and a sequence of changes in electrophysiological properties occurs during normal development of chick embryonic myocardial cells in situ (*2–4*). Up to 4 days in ovo, a time when the heart tube is undergoing complex morphogenetic movements, the young myoblasts lack fast Na^+ channels (*2–4*). This is indicated by the fact that the young cells retain slow rates of rise when the membrane is hyperpolarized to a take-off potential of –80 mv. Their action potentials are generated by kinetically slow Na^+ channels, which confer slow maximal rates of rise ($+\dot{V}_{max}$) (usually less than 25 volt/sec) and which are insensitive to tetrodotoxin (TTX), a blocker of fast Na^+ channels (Fig. 1, A and B). Sensitivity to TTX first appears on about day 5, marking the initial appearance of fast Na^+ channels (*2–4*). From day 5 to day 8, the action potentials have intermediate rates of rise (30 to 80 volt/sec) and partial sensitivity to TTX; that is, TTX reduces $+\dot{V}_{max}$ to a low value (5 to 10 volt/sec), about the same as that found in young cells. The inward current during these residual action potentials must be carried by slow Na^+ channels, which are not blocked by TTX. Thus, the membrane contains both TTX-sensitive fast Na^+ channels and TTX-insensitive slow Na^+ channels at this stage of development. After day 8, TTX completely abolishes all excitability despite intense stimulation (Fig. 1, C and D), indicating that the density of functional slow Na^+ channels has diminished. The $+\dot{V}_{max}$ values increase progressively to about 150 volt/sec by day 18, suggesting the acquisition of an increased density of fast Na^+ channels. The resting potential also increases gradually during development from about –35 mv on day 2 to about –80 mv by day 12 (*2–4*). The increase in resting potential coincides with a decrease in the incidence of pacemaker activity. Both of these changes can be accounted for by an increase in K^+ permeability (P_K) during development (*2, 5*).

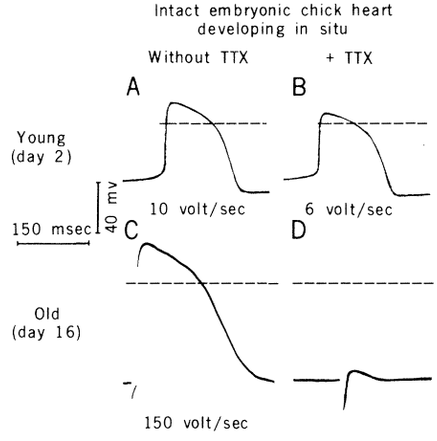

Fig. 1. Electrophysiological properties of young (day 2) (A and B) and old (day 16) (C and D) chick embryonic hearts developing in situ. (A) Control action potential with slow (< 20 volt/sec) $+\dot{V}_{max}$ and with pacemaker potential. (B) After addition of TTX (1 μg/ml), the action potential rate of rise and overshoot are unchanged. (C) Rapidly rising recording ($+\dot{V}_{max}$ of 150 volt/sec) from ventricular cell with a high resting potential (–80 mv). Note absence of pacemaker potential. (D) Tetrodotoxin (0.1 μg/ml) abolished all excitability despite intense field stimulation. Calibrations apply throughout. The horizontal broken line gives zero potential. Extracellular electric field stimulation was given in (C) and (D) (note artifacts).

The normal sequence of membrane electrical differentiation can be interrupted in vitro. Sperelakis and co-workers (*6*) have shown that development is arrested at the stage reached at the time of explantation when hearts or fragments of hearts are placed in organ culture. For example, young hearts retain their high density of TTX-insensitive slow Na^+ channels and fail to gain fast Na^+ channels in vitro.

When young embryonic cells are cultured as monolayers or as reaggregates, they do not further differentiate; that is, they retain characteristically young properties, including TTX-insensitive slowly rising action potentials and pacemaker activity (*7*) [although limited development may be achieved in some cells (*8*)], as found in the case of organ-cultured young hearts. When cells are dissociated from old embryonic hearts (14 to 20 days in ovo) with trypsin and subsequently surface-cultured as monolayers, the electrical properties revert to resemble those of the young heart; that is, the cells lose their TTX-sensitive fast Na^+ channels, regain a high density of slow Na^+ channels, and resume pacemaker activity (*3, 9*). This reversion can be partially prevented by culture in elevated K^+ (*10*), and complete retention of highly differentiated electrophysiological properties in vitro can be achieved in spherical reaggregates (*11*).

Niu and co-workers (*12*) have reported the induction by adult chicken heart RNA of spontaneously beating tubes and sheets of cardiac cells in cultured postnodal pieces of chick blastoderm; the effect became apparent about 5 days after RNA treatment. We have recently corroborated these results and have recorded typical cardiac action potentials from these cells (unpublished observations). The induction of cardiac tissue in undifferentiated postnodal pieces indicates that determination, as well as differentiation, can be produced in vitro. This led us to test whether cultured young embryonic myocardial cells in organ-cultured hearts and in spherical reaggregate cultures could be induced to develop fast Na^+ channels by treatment with adult heart messenger RNA (mRNA) in vitro. As indicated by rapidly rising action potentials and TTX sensitivity, we conclude that such induction does occur.

For organ culture, spontaneously con-

tracting heart tubes were removed from 48- to 60-hour (Hamburger and Hamilton stage 12 to 17) chick embryos, and the ventricular regions were cultured in plastic tubes with Rinaldini culture medium. Spherical reaggregate cultures were prepared from tubular hearts (48 to 60 hours) by methods described previously (*11*). The mRNA was prepared from adult chicken hearts by methods presented elsewhere in detail (*12, 13*). Messenger RNA (0.1 ml from a stock solution of 80 absorbance units per milliliter) was added to the media of the organ-cultured hearts and the reaggregate cultures. The media were not changed before impalements. The results reported here were repeated three times with three different preparations of mRNA.

Standard intracellular microelectrode techniques were used to examine the electrophysiological properties of the culture preparations. Cells impaled in the organ-cultured (days 5 to 14) young embryonic hearts possessed slowly rising TTX-insensitive action potentials with pacemaker potentials (Fig. 2, A and B) and accompanying contractions, resembling young intact hearts developing in situ (Fig. 1, A and B). The average takeoff potential in the cultured cells was -46 ± 2 mv (mean $\pm$ 1 standard error) ($N = 11$), and the maximal rates of rise were slow (5 to 10 volt/sec); hyperpolarization by current injection did not increase $+\dot{V}_{max}$. However, when incubated with heart mRNA for 10 days, the average resting potential increased to -64 ± 3 mv ($N = 29$), and $+\dot{V}_{max}$ increased to 79 ± 4 volt/sec. Further experiments were performed to determine the time course of the effect: less than 5 days was insufficient time for the induction to become evident, but by day 6, and as late as day 18, the effect was prominent. In most cells, TTX (0.1 μg/ml) completely abolished all excitability despite intense extracellular field stimulation (Fig. 2, C and D), and contractions ceased simultaneously. Only 2 cells out of the 29 sampled were partially sensitive to TTX. These cells had slow rates of rise (20 volt/sec) initially, and were found in a single heart which was exposed to half of the usual mRNA concentration. Pacemaker activity was absent in all cells penetrated. Chronaxie (determined from strength-duration curves), a measure of excitability, was 0.7 and 1.4 msec for two hearts. In addition, the cells in organ-cultured hearts produced overshooting slowly rising electrical responses on addition of isoproterenol ($10^{-6}M$) (after TTX blockade of the action potentials), similar to those found in differentiated hearts (*14*).

The mRNA-induced changes in membrane properties of organ-cultured hearts were prevented by addition of cycloheximide (1 μg/ml) at the time of mRNA addition. The cells treated with mRNA plus cycloheximide retained slowly rising TTX-insensitive action potentials with pacemaker potentials, similar to those of the controls. Prevention of the mRNA inductive effect by cycloheximide suggests that protein synthesis is required in the mediation of the effects. Organ-cultured hearts treated with liver mRNA did not gain fast Na^+ channels. Pretreatment of the heart mRNA with purified ribonuclease destroyed the inductive capacity of the exogenous mRNA in two preliminary experiments.

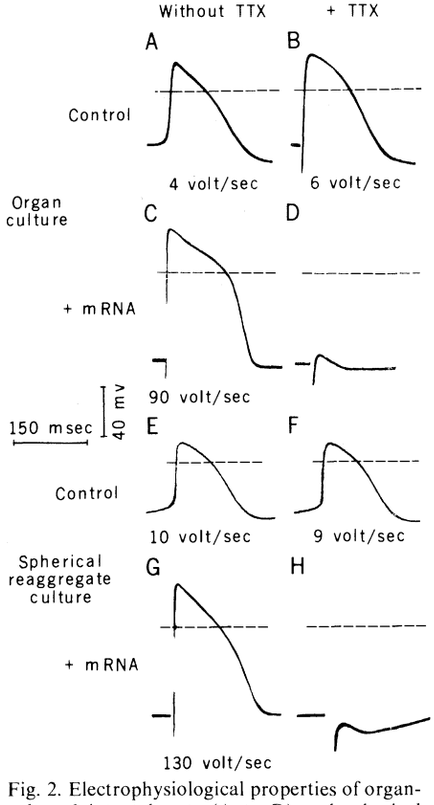

Fig. 2. Electrophysiological properties of organ-cultured intact hearts (A to D) and spherical reaggregate cell cultures (E to H) of young (day 2.5) embryonic chicken heart myoblasts. (A) Control recording from organ-cultured tissue. (B) Action potential unaltered after addition of TTX (1.0 μg/ml). (C) Recording from mRNA-treated organ-cultured heart showing fast rate of rise (90 volt/sec) and higher resting potential. (D) Complete blockade of excitability in the same cell by TTX. (E) Control recording from one cell in a spherical reaggregate culture. (F) Tetrodotoxin (1.0 μg/ml) had no effect on the action potential rate of rise or overshoot. (G) Recording from one cell in a mRNA-treated reaggregate cell culture showing a fast rate of rise (130 volt/sec) and a high resting potential (-75 mv). (H) Tetrodotoxin (0.1 μg/ml) rapidly abolished the action potential in this cell. Calibrations apply throughout. The horizontal broken line gives the zero potential. Electric field stimulation was applied in (C), (D), (G), and (H).

Results obtained from spherical reaggregate cell cultures were similar to those of organ-cultured tissue. Recordings from control cells (Fig. 2, E and F) showed slowly rising TTX-insensitive action potentials with pacemaker potentials and relatively low resting potentials (-42 ± 1 mv) ($N = 5$). Incubation with heart mRNA resulted in the cells possessing rapid rates of rise (up to 130 volt/sec), complete sensitivity to TTX, and high resting potentials (-70 ± 2 mv) ($N = 6$) (Fig. 2, G and H). Aggregates treated with cycloheximide plus mRNA failed to develop further, as found for the organ culture experiments. Incubation with liver mRNA did not bring about the changes in membrane properties. The contractions of aggregates treated with heart mRNA were abolished by TTX, despite increased stimulus strength; control reaggregates continued to contract in the presence of TTX.

The results show that mRNA isolated from adult chicken hearts can bring about the appearance of fast Na^+ channels in cultured young embryonic chick cardiac myoblasts that possessed only slow Na^+ channels at the time of culturing. Without mRNA, differentiation did not proceed. The normal sequence of development in situ involves the appearance of fast Na^+ channels on day 5 and a gradual increase in the number of such channels thereafter. Therefore, we think that the appearance of fast Na^+ channels in mRNA-treated hearts, as judged by relatively rapid rates of rise and complete sensitivity to TTX, represents induction of further differentiation in vitro. The loss of pacemaker activity observed in mRNA-treated cultures also served as an index of induced differentiation. These findings suggest that the arrested development observed in culture is due to the absence of extrinsic factors that are normally present in situ.

The inductive mechanism is not known—for example, whether the mRNA is acting on the cell surface or intracellularly. One possibility is that the exogenous mRNA is taken up into the cultured cells, as demonstrated previously in other in vitro systems (*15*) and then, either through the agency of cytoplasmic messengers or by entry into the nucleus, exerts a genotropic influence. This, in turn, could result in the synthesis of new proteins, which enter the cell membrane to confer fast Na^+ channel activity and an increase in P_K. McDonald *et al.* (*16*) reported that cycloheximide prevented desensitization to TTX—namely, blocked the appearance of TTX-insensitive slow channels in young reaggregate cell cultures. This is different from the results reported here, in which protein synthesis has been associated with

the production of TTX-sensitive fast Na^+ channels. However, one similarity between these two studies is the effect of protein synthesis on membrane channels. There is evidence that the changes induced by exogenous mRNA are stable (*17*); that is, once the messenger has been removed, either by degradation or by changing the medium, the induced alterations persist. Alternatively, translation of the exogenous messenger taken up may lead to synthesis of stable membrane proteins with a low turnover rate, mimicking a stable genetic alteration (*18*). Although the mRNA used here is heterogeneous, one messenger must control synthesis of fast channel protein, while another could affect P_K, resulting in the observed increase in resting potential.

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BCG Inhibition of Murine Leukemia: Local Suppression and Systemic Tumor Immunity Require Different Doses

Abstract. *The quantitative relationships between bacillus Calmette-Guérin (BCG) and tumor cells which are optimal for suppressing the growth of tumor cells in BCG–tumor cell mixtures are detrimental to the development of a sustained, systemic tumor rejection immunity in the LSTRA murine leukemia.*

The bacillus Calmette-Guérin (BCG) strain of *Mycobacterium bovis* has become a popular agent for immunotherapy of cancer (*1*). Its action may involve combinations of several mechanisms: (i) generalized ("nonspecific") stimulation of immune responses and lymphoreticular activity, following systemic administration (not in proximity to tumor cells) (*2*); (ii) local, nonspecific destruction of tumor cells at the site of a delayed hypersensitivity response to BCG ("innocent bystander" effect) (*3, 4*); and (iii) true adjuvant effect, wherein administration of BCG in temporal and spatial proximity to tumor cells (either mixed in the same inoculum or by infiltration of tumor nodules) results in augmented development of systemic, tumor-specific rejection immunity (*5, 6*). In the treatment of human cancer, the most reliable effect of BCG has been the local destruction of tumor nodules into which the material has been injected (*7*). Systemic effects, suggestive of augmented tumor rejection immunity, have been rare, although they are the ultimate goal of immunotherapy (*8*).

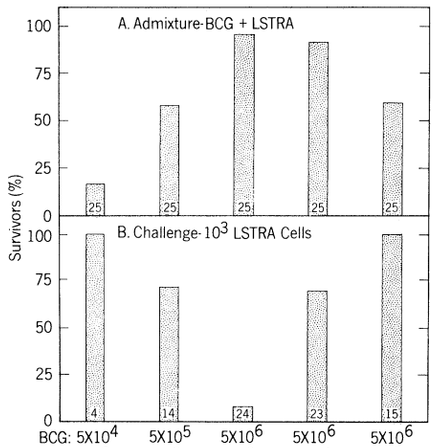

Fig. 1. Survival of mice that received intradermal injections of admixtures of LSTRA cells and BCG (pooled data of two experiments). (A) Proportion of mice in which growth of the LSTRA cells in the admixture was suppressed. (B) Proportion of mice (survivors of the admixture injections) that rejected a challenge injection of 10^3 LSTRA cells given intradermally 42 days after injection of the admixtures (all challenge control mice died). Numbers in the bars represent the total number of mice in each group.

Little is known about the dose-response relationships of the BCG-initiated antitumor responses. In most systems diminution of BCG dose eventually eliminated the immune stimulant effects (*9*). However, under certain circumstances high BCG doses have interfered with antitumor effects (*10*), while in a chemoimmunotherapy model, BCG was effective at doses as low as 800 viable organisms (*11*). Clinical dosimetry has been largely empirical. The highest tolerated dose is sought, and tends to be limited more by the concentration of the vaccines available than by biologic considerations. We report here that in one animal model the doses of BCG and tumor cells which regularly produced local suppression of tumor growth did not induce detectable tumor immunity, while the doses that were required to induce tumor immunity were relatively ineffective in achieving local suppression of tumor growth.

In this study, local tumor suppression was evaluated by the growth or suppression of tumor cells mixed with BCG before injection (*3*); adjuvant effects were assessed by the resistance to tumor challenge in mice which had suppressed the original tumor-BCG inoculum (*5*). The LSTRA Moloney virus–induced leukemia (*12*), of BALB/c origin, was studied in male $(BALB/c \times DBA/2)F_1$ hybrids (*12*). The tumor was maintained by weekly intraperitoneal passage of ascites tumor (0.2 ml of a 1 : 1000 dilution of ascites fluid in minimum essential medium, usually containing 2×10^4 to 4×10^4 viable tumor cells). Intradermal inoculation of 10^3 cells results in greater than 95 percent mortality, and