Deficits in Elevating Membrane Potential of Rat Fibrosarcoma Cells after Cell Contact

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ABSTRACT

Most cancer cells are known to have lower resting cellular potentials than do their normal counterparts. This study investigates how these potentials establish themselves during growth and cellular contact in tissue culture. Normal quail embryonic fibroblasts and quail fibrosarcoma (QT-35) and normal rat kidney cells and rat fibrosarcoma (from rat fibroblasts chemically transformed by nitroquinoline oxide) were recorded intracellularly using high-impedance micropipets.

In high-density high-contact cultures, both quail and rat cancer cells had lower potentials than did normal cells (−20.7 compared to −40.1 mV for quail and −30.7 compared to −61.9 mV for rat).

In low-density mitotically synchronous cultures, the rat cells were recorded every 4 hr for 96 hr. Starting at a low density, normal cell membrane potential is maintained at a low level through subsequent cell divisions. Without any additional change in cell density, the potential suddenly elevates to a high level. The membrane potential of cancer cells is, by contrast, unrelated either to cell density or to time. Cancer cells maintained an intermediate potential from low to very high densities and never elevated their potential to high values. The failure of cancer cells to reach high potentials may be linked to their uncontrolled cell division.

INTRODUCTION

Several studies have established that cancer cells have a smaller resting cellular potential (voltage) when compared with their normal counterparts. Lash et al. (11) in 1955 first described reduced potentials in carcinoma of the cervix compared to normal cervix. Tokuoka and Morioka (25) found the same in gastric mucosa and gastric carcinoma as did Limberger (12) in liver and hepatoma. Balitsky and Shuba (2) found large differences in muscle and rhabdomyosarcoma and Jamakosmanovic and Loewenstein (8) found significant differences in normal and cancerous rat thyroid. Schanze's (21) early study is one of the few which do not support the notion of increased normal cell polarity, but his data lack comparison between matched tissue types. The question arises as to whether there is a functional relationship between these voltage differences and the active control of growth as was first proposed by Cone (6) in 1971. More recently, Binggeli and Cameron (3) found significantly reduced potentials in fibrosarcoma and hepatoma in vivo as compared to comparable normal rat tissues. Picker et al. (17) found normal-cancerous differences in astrocytes to be insignificant in well-differentiated astrocytoma but very large in glioblastoma multiforme.

Several authors have shown a correlated change in membrane potential with either initiation or cessation of growth. Kiefer et al. (9) found that the transition from resting state to mitotic activity was marked by an early significant decrease in plasma membrane potential. Cone and Tongier (7) showed that the cell membrane potential increased with time and cell density up to contact "inhibition" in tissue cultures of normal 3T3 cells. Adam et al. (1) have also shown increases in potential coinciding with the formation of intercellular contacts and the emergence of cell density-dependent inhibition of growth. Martz and Steinberg (13) using 3T3 cells and Schmialek et al., (23) using embryonic hamster cells found that cells grew for one additional generation after cell contact and then ceased.

How do these different potentials establish themselves in normal and cancer cells, and how might they either correlate with or cause growth changes? This study first investigates the cell potential of both normal and cancerous cells in confluent culture to verify the difference in potential. Changes in potential are investigated in synchronized seeded populations in culture with increases in time, density, and cell contact.

MATERIALS AND METHODS

Cells and Culture Methods. Four cell types were used in this study: primary normal rat kidney epithelial cells and rat fibroblasts chemically transformed with nitroquinoline oxide and transplanted into rats as a fibrosarcoma [Rasheed et al. (18)]; and normal quail embryonic fibroblasts and quail fibrosarcoma chemically transformed in vivo into fibrosarcoma and classified according to the method of Moscovici et al. (14) (QT-35). The cells were maintained in flasks, grown to confluence, trypsinized, and split in 1:5 ratios, and the media were changed every 3 days.

The rat cells were lightly trypsinized and shaken to dislodge only dividing cells in order to achieve a synchronous cell population. They were seeded at a density of 1 × 10⁶ cells/35-mm plastic Petri dish (1 × 10⁶ cells/sq cm) and cultured in Hank's balanced salt solution supplemented with glutamine, amino acids, vitamins, and 10% fetal calf serum (Figs. 1 and 2). The medium was not changed during the period of this study. Normal rat cells were seeded occasionally after trypsinization in small clumps (uncounted) in 55-mm Petri dishes.

The quail cells were trypsinized, and all cells were used as an asynchronous population and seeded at 2 × 10⁶ cells/55-mm dish (6.4 × 10⁶ cells/sq cm). The normal quail cells were cultured in Eagle's basic medium supplemented with 2% fetal calf serum. The QT-35 cells were grown in Ham's F-10 solution with amino acids, vitamins, glutamine, 10% fetal calf serum, 1% chicken serum, and 10% tryptose phosphate broth (Figs. 5 and 6).

Recording Methods. During the electrical recording sessions, the cells were maintained in their dishes at a constant 37°C in a constant-temperature hollowed aluminum heating block and were gassed with flowing warm, humidified 95% air:5% CO₂. The cells were observed constantly under phase-contrast microscopy and were penetrated with high-impedance glass micropipets (50 to 200 megohm; tip potentials, <5 mV) filled with 3 M KCl, and monitored visually. The micropipets were

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led through silver:AgCl agar into a high-impedence amplifier, and a
silver:AgCl agar electrode was placed in the cell medium. Great care
was exercised to maintain the recording situation with a minimum of
vibration, movement, and electrical noise, the latter by means of a
Faraday cage.

It had been found in our laboratory that temperature, humidity, pH,
electrical noise, electrode size, resistance, and vibration could have a
devastating effect on cell potentials. The cell recordings had to produce
stable potentials for the generally accepted time of 20 sec without visible
morphological change in order to be acceptable.

Protocol. Recordings of membrane (cellular) potential were done first
from normal and transformed cells of both quail and rat in confluent
monolayers in order to form a basis of comparison with other cells in
the literature and to form a base line from which to study changes in potential
with time and increasing density.

Both rat (Figs. 3 and 4) and quail cells (Figs. 5 and 6) were grown to
confluence, and after 24 hr recordings were done in 3 to 5 cells from
each dish to make the total number of cells. All recordings were made
more than 24 hr after the addition of medium. The rat cells were then
selected from another experiment so as to look for potential changes
with time and density in synchronized cultures. These were seeded in
low density (8 to 9 x 10^4 cells/sq cm), and cell potentials were recorded
after the first hr and then every 4 hr for 70 to 90 hr. Three to 5 cells
were recorded for each of 3 dishes for each cell type at each time (e.g.
see Figs. 1 to 4). The medium was not changed during the course of
this experiment. Cell densities were determined by direct counting of a
sample of photomicrographs. Cell overlap did not pose a problem
throughout most of the time considered. Membrane potentials were also
recorded from normal rat kidney cells after being seeded in clumps of
various sizes.

RESULTS

Chart 1 presents the frequency distribution of the potentials
of quail cells, both normal and transformed in confluent monolayer (3.9 x 10^4 and 6.0 x 10^4 cells/sq cm, respectively). Both
cell types have somewhat normal distributions that are almost
separate and exclusive, with the transformed type showing
lower potentials. Chart 2 shows essentially the same pattern for
the confluent rat kidney cells (approximately, 2.7 x 10^4/sq cm)
and rat transformed fibroblasts (approximately, 3.5 x 10^4/sq cm)
but with somewhat higher values for both cell types. Table 1

presents the statistics for all 4 cell types, both quail and rat, and
demonstrates that within each species the differences between
these normal and transformed cells are highly significant (p <
0.001).

In order to study the development of the membrane potential
over time, two types of cells were followed for 4 days. Normal
rat kidney cells and chemically transformed rat fibroblasts (NQ
cells) were used. They were initially seeded as dividing cells in
low density and followed through their growth phase up to and
beyond confluence. Membrane potentials were recorded every
4 hr for both populations of cells, and the results are presented
in Chart 3. Both cell types started with a potential at approxi-
mately the same low level (−8 to −10 mV) and begin a modest
increase over the next 10 hr. At this point, the membrane
potential of the normal cells leveled off (at approximately −17
mV) and maintained themselves for the next 20 to 30 hr at that
value. The potential of the fibrosarcoma cells continued to in-
crease after 10 hr, and exceeded the normal values at this time
period by 10 to 15 mV for the next 20 to 30 hr and continued
unchanged indefinitely. The normal cells made a significant break
at about 40 hr and dramatically increased their potential by 3-
or 4-fold. They rose to a terminal value of over −60 mV at about
the 50th hr and maintained themselves for the duration of the
experiment.

These membrane or cellular potential values can be compared to
 cellular density (in cells per sq mm) within the 2 cell types as
illustrated in Chart 4. Since the cells were seeded primarily as
mitotic cells, the densities tended to remain constant for several
hr and abruptly, nearly synchronous with cell division, began to
rise to a new somewhat constant level. The tumor cells seemed
to have a cell cycle about 18 hr long and had their first and
second division at about 18 and 36 hr, respectively. The normal
kidney cells had a somewhat longer cycle time, dividing at about
23 and 46 hr, respectively. The notable and not unexpected
difference was that the tumor cells entered into a third division
and continued dividing well after that to form dense mounds of
cells that could not be visually counted, while the normal cells
ceased dividing after the second division and formed the usual
stable confluent monolayer.

It can be seen that the normal kidney cells underwent their
rapid increase in membrane potential somewhere between the
38th and 45th hr (Chart 3) and their last cell division somewhere
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Table 1
Summary of cellular potentials and statistics from each of the 4 cell types recorded in confluent monolayer

<table>
<thead>
<tr>
<th>In vitro cell type</th>
<th>Mean cellular potentials (mV)</th>
<th>S.D.</th>
<th>S.E.</th>
<th>No. of cells</th>
<th>p value of difference in means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quail fibroblasts (QT-35)</td>
<td>−40.1</td>
<td>8.89</td>
<td>1.00</td>
<td>78</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quail fibrosarcoma QT-35</td>
<td>−20.7</td>
<td>5.96</td>
<td>0.54</td>
<td>120</td>
<td></td>
</tr>
<tr>
<td>Rat kidney</td>
<td>−61.9</td>
<td>8.89</td>
<td>0.81</td>
<td>119</td>
<td></td>
</tr>
<tr>
<td>Rat fibrosarcoma</td>
<td>−30.7</td>
<td>7.36</td>
<td>0.46</td>
<td>255</td>
<td></td>
</tr>
</tbody>
</table>

Charts:

Chart 3. Cellular potentials (in mV) of normal (○) and transformed (●) rat cells measured over time (hr) after seeding of synchronized mitotic cells at 1 × 10^6 cells/55-mm dish. Data points, means of a total of 10 cells from 4 different dishes.

Chart 4. Cellular density (cells per sq mm) of normal (○) and transformed (●) rat cells over time (hr) after seeding of synchronized mitotic cells. Data points, mean densities of 4 photographed areas from 4 dishes.

Chart 5. Recorded membrane potentials of cells plotted against the number of cells in the contact group for both normal (NRK) cells and transformed (QO) cells.

Chart 6. Cellular potentials (in mV) of normal rat kidney cells that were seeded in large clumps. Data points, mean values from 10 cells within a large clump at each time period.

between the 40th and 55th hr (Chart 4). It may be that the increase in potential took place just before the final division. In the case of the tumor cells, there does not seem to be any correlation between the rise in membrane potential and the occurrence of periods of cell division, there being more than 2 divisions after the final potential of −30 mV is set.

Some cell cultures were seeded with cells only partially disaggregated with trypsin resulting in cell groups with varying size.

There were individual, isolated cells, small groupings of 2, 3, and 4 or more cells and larger aggregates. After 16 to 24 hr, recordings were done with attention to the size of the aggregate. These results are plotted in Chart 5 (in both types of cells the smaller cell groupings were precisely counted, but as the groups grew to 40 or 50 cells counting became more and more difficult and was no more than ±10% accurate.) Each of the data points represents the recording of membrane potential of 20 to 70 cells in each size of cell aggregate.

It can be seen clearly that the membrane potential started from a low point in both the normal and tumor cells and increased with the increase in cell clump size. In the rapid-growth segment, the membrane potential of the normal cells increased about 3
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Discussion

Lower potentials (voltages) were found in transformed quail and rat cells (~21 and ~31 mV, respectively) than in 2 types of normal tissue cells (~40 and ~62 mV). This confirms findings about cancer and normal cells in vivo produced by Lash et al. (11) in cervix, by Tokuoka and Morikoka (25) in stomach, by Limberger (12) in liver, by Baittsky and Shuba (2) in muscle, by Picker et al. (17) in glia, and by Binggeli and Cameron (3) in liver and connective tissue. Similar results were obtained in vitro by Jamakosmanovic and Loewenstein (8) in thyroid and 12 different types of thyroid tumor cells. These reinforce the notion that cancer cells or transformed cells have lower potentials than do their normal counterparts in particular and normal cells in general.

A few exceptions have been described, notably by Schanne (21) who found a few medium potentials for some tumors and a few lower potentials for normal fibroblasts. Picker et al. (17) also described a well-differentiated astrocytoma with a high potential (equal to its normal cell counterpart). Boonstra et al. (4) found moderate potentials in neuroblastoma in some phases of the cell cycle but not in others.

With these few exceptions, there is an overall consistency which may perhaps be considered a generalized phenomenon, a phenomenon to which Cone (6) pointed over a decade ago.

It has been known that maximum membrane potential was achieved at confluence. The preconfluent potentials were always low and were usually values that were ignored or only used as an indicator of nonconfluence. The patterns of growth in potential in this study have shown that following seeding of mitosing cells, the potential was very low and then rose slowly to an intermediate value that endures throughout the next period of mitosis. Normal and cancer cells then show different characteristic patterns of development of membrane potential as the population proceeds to confluence.

Normal Cells. In normal cells in the first few hours in a nonconfluent freshly seeded synchronous culture (the individual cells having just divided), there was an initial small increase in potential. This small increase may have been the result of recovery from light trypsinization, shaking detachment, or repletting of the mitosing cells. The cells in this initial period were flattening and possibly establishing initial contact. There was no further change in potential during the next 30 hr. Any increase in cell contact did not have any effect on potential.

The first synchronous mitosis took place at about the 23rd hr without significantly affecting the potential baseline. If short-acting changes in potential were to take place, they might be missed in these experiments because of the relatively long sampling time of 4 hr. A doubling of the cell number and accompanying increases in cell contact did not significantly affect potential around the time of mitosis.

The timed sequence of events that occurred between the 35th and 55th hrs was the most revealing in terms of suggesting possible mechanisms of growth inhibition. Starting with a constant unchanging density (150,000/sq cm) that at least permitted liberal cell contact, cell voltage suddenly increased from a previous lower and rather stable value. This demonstrates that increased cell density per se, although possibly a necessary condition for increased voltage, is certainly not a sufficient one.

Shortly following the very large change in potential, the second and final mitosis occurred. The timing of this second mitosis was almost exactly twice the time of the first. The cell cycle time then was the same for the first 2 divisions apparently independent of either cell density or potential. Having one more mitosis after cell contact confirms the findings of both Martz and Steinberg (13) and Schmialek et al. (23) in embryonic hamster cells. The occurrence of this mitosis suggests that if a high membrane potential is a necessary condition for mitotic inhibition it is not a sufficient condition.

After this second mitosis, the potential remained at a high level and no more mitoses occurred. The population was "density" or "contact" inhibited. This correlation between high potential and contact inhibition was also observed by Adam et al. (1) and Cone and Tongier (7).

If there is this correlation between high membrane potential and inhibition of cell division how can the presence of the second mitosis after the increase in potential be explained? A possible explanation might be that the higher potential may have an inhibiting effect on DNA synthesis. The second mitosis then would have occurred because DNA synthesis had been accomplished during the previous period of low potential. Once the potential was elevated, however, subsequent synthesis would have been inhibited and any further mitosis thus prevented.

Cancer Cells. In confluent freshly seeded synchronous populations of cancer cells, the density remained constant for 18 hr while the potential rose steadily from a very low value of ~10 mV to a moderately low value of ~31 mV. The question arises as to why the membrane potential of unconnected single cancer cells rose above the early low potential of the normal cells. Certainly this is an early indication that cancer cells have an altered membrane structure. Later in time, the relation between normal and cancer cell potential reversed, and the normal cells had a much higher value than did cancer cells.

The cancer cell potential stabilized at ~31 mV. Mitoses continued from first to second to third division. The cellular potential did not change during any of the divisions. The cells simultaneously came into close contact after the second division, and this also had no effect on the potential. Divisions continued beyond the third, but the cells became more difficult to count. Cell division was not inhibited; therefore, density continued to rise while the potential continued at this constant level indefinitely or until cell death occurred due to overcrowding.

Sachs et al. (20) and Boonstra et al. (4) both recorded membrane potential fluctuations during the cell cycle showing that the potential peaked at the conclusion of DNA synthesis and fell during mitosis, with a gradual recovery during G and S phases. Sachs et al. (20) concluded that membrane potentials play no role in mitotic regulation; however, they recorded from isolated single cells only. Had they continued the recording to the point of contact inhibition, they may have found an entirely different result. In addition, in confirmation of our findings, all membrane potential values of isolated cells in that study were below ~32 mV.

Our data for changes in the normal cell potential over time compare very well with those of Cone and Tongier (7) which
were recorded from Chinese hamster ovary cells. We have replotted their numerical data in Chart 7 in order to compare their results directly with our own. The replotting shows a long period of low potential at around -10 mV followed by a sudden rapid rise at about 35 hr to -60 mV. Their rising phase presents earlier, no doubt because of the greater cell concentration (density) at seeding. Cone stated that the basis of contact inhibition in monolayer populations in vitro at confluency is the result of potential level mediation through cell surface contact.

When the normal cells were seeded in clumps (partially separated) (Chart 5), the potential in these clumps rose much more rapidly than that of the cells seeded well separated. Undoubtedly, many cell-to-cell contacts were maintained during the seeding process, and the groups were able to elevate potential to high levels in proportion to their clump size (Chart 6).

In both normal and cancer cells seeded as clumped-cell groups, the relation of cell group size to potential is direct and almost linear after the groups are beyond a certain minimum size. After that point, the normal cells showed a 3-fold greater tendency to increase potential with increased number of cells (Chart 6). In other words, the addition of new cells to a normal cell clump was 3 times more effective in elevating the membrane potential than it was in cancer cells. This feature strongly points to the quality of cell-to-cell contact as a significant factor in explaining this difference between normal and cancer cells.

Lower membrane potential, in particular, may initiate mitotic activity as Kiefer et al. (9) described. Altered membrane potentials may be correlated with changes in cell-to-cell junctions. Jamakosmanovic and Lowenstein (8), Rose et al. (19), and Borek et al. (5) have shown that normal cells in contact with each other have membrane channels between themselves that permit the passage of small- and medium-molecular-weight molecules including high permeability inorganic cations. This, in part, gives rise to some uniformity of potential between cells that have established such junctions. The aforementioned investigators found that many but not all cancer cells do not establish these connections to any detectable degree with either themselves or normal cells, are electrically more isolated from one another, and have lower membrane potentials. By contrast, normal cells show ionic coupling, contact inhibition, and gap junctions simultaneously. Schindler et al. (22) found in cervix that gap junctions virtually disappeared during transformation from normal to dysplastic to carcinoma cells. Stomatologiou and Marshall (24) found that the appearance of gap junctions correlated well with density dependent inhibition of growth in normal cells.

Weinstein et al. (26), looking at various junctions in several cancer cells found that of 17 cancer cell types, 7 had reduced or absent gap junctions and 4 had reductions in other types of junction. As he warned, many studies are qualitative and not quantitative. Alterations in junctions may take place in frequency, quality, or size and not just presence or absence. Pauli and Weinstein (16), for example, found an absence of only one of 2 normal types of gap junction in one carcinoma cell. Larsen (10) described the presence of gap junctions in several normal and cancer cells but also described large size differences between them. Murray et al. (15) described large differences in the number of gap junctions per cell among SW-13 adenocarcinoma cells in culture, depending on whether the cells were within the monolayer or rounded and separating from it.

Clearly, cancer cells show a variety of junctional relationships with no universal pattern present. However, there are sufficient studies suggesting that significant changes are present in many cell types. Our studies point to a significant deficit in membrane potential in one more cell type and perhaps to deficits in cell-to-cell contact. Further studies need to define how general these phenomena are among different cancer types and whether they point to possible mechanisms of growth control.

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Fig. 1. Synchronized normal rat kidney cells 1 hr after seeding at 1 x 10⁶ cells/dish (800 cells/sq mm; 80,000 cells/sq cm). Phase contrast, x 200.

Fig. 2. Synchronized transformed rat fibroblast cells 1 hr after seeding at 1 x 10⁶ cells/dish (950 cells/sq mm; 95,000 cells/sq cm). Phase contrast, x 200.

Fig. 3. The same synchronized normal rat kidney cells at 40 hr after seeding just at the point of confluence (1,600 cells/sq mm; 160,000 cells/sq cm). Phase contrast, x 200.

Fig. 4. The same transformed rat fibroblast cells at 40 hr after seeding just at confluence (2,000 cells/sq mm; 200,000 cells/sq cm). Phase contrast, x 200.

Fig. 5. Embryonic quail fibroblasts at confluence. Phase contrast, x 200.

Fig. 6. Transformed quail fibroblasts (Q-T 35) at confluence. Phase contrast, x 200.
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