

Purging of the Neuroblastoma Stem Cell Compartment and Tumor Regression on Exposure to Hypoxia or Cytotoxic Treatment

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Abstract

We worked out an experimental protocol able to purge the stem cell compartment of the SH-SY5Y neuroblastoma clone. This protocol was based on the prolonged treatment of the wild-type cell population with either hypoxia or the antiproliferative etoposide. Cell fate was monitored by immunocytochemical and electrophysiologic (patch-clamp) techniques. Both treatments produced the progressive disappearance of neuronal type (N) cells (which constitute the bulk of the tumor), leaving space for a special category of epithelial-like substrate-adherent cells (S₀). The latter represent a minimal cell component of the untreated population and are endowed with immunocytochemical markers (p75, c-kit, and CD133) and the electrophysiologic "nude" profile, typical of the neural crest stem cells. S₀ cells displayed a highly clonogenic potency and a substantial plasticity, generating both the N component and an alternative subpopulation terminally committed to the fibromuscular lineage. Unlike the N component, this lineage was highly insensitive to the apoptotic activity of hypoxia and etoposide and developed only when the neuronal option was abolished. Under these conditions, the fibromuscular progeny of S₀ expanded and progressed up to the exhaustion of the staminal compartment and to the extinction of the tumor. When combined, hypoxia and etoposide cooperated in abolishing the N cell generation and promoting the conversion of the tumor described. This synergy might mirror a natural condition in the ischemic areas occurring in cancer. These results have relevant implications for the understanding of the documented tendency of neuroblastomas to regress from a malignant to a benign phenotype, either spontaneously or on antiproliferative treatment. [Cancer Res 2007;67(6):2402-7]

Introduction

We carried out a program of research aimed at revealing the process of cell renewing in neuroblastoma, a tumor of childhood characterized by an unpredictable clinical course and a remarkable plasticity, resulting in multiform phenotypes (1, 2). A peculiar feature of this tumor is the tendency to regress from a malignant to a benign phenotype (ganglioneuroma or, rarely, a neurofibromatous mass; ref. 3).

Our approach to this problem was based on the use of a human neuroblastoma adrenergic SH-SY5Y clone (henceforth referred to as SY5Y), taking advantage of our previous studies (2), which unraveled

the renewal of this tumor by an integrated investigation, which coupled classic immunocytochemical procedures with sophisticated electrophysiologic measurements done with the patch-clamp technique. The latter permits quantitative measurements of the clusters of ion currents expressed on the surface of single cells, providing a powerful tool to identify cell types and differentiation stages. In this approach, it was possible to use the grouped currents in the same way as cluster of differentiation (CD) antigens are widely used in cell biology and immunology (4, 5). By analogy, we introduced the term electrophysiologic cluster of differentiation # (ECD#) to describe the quantitative expression of a group of ion channels on the cell under investigation, where # is a signal identifying a determinate stage of differentiation along a specific pathway (2).

The aim of this work was to purge and characterize the neuroblastoma stem cells, taking advantage of the reported higher sensitivity to apoptotic stimuli of the neuronal (N) as contrasted with the staminal cell compartment. To this end, we exposed the SY5Y cell population to treatments eliciting apoptosis via p53 protein activation, already reported on SY5Y (6-8), i.e., etoposide and hypoxia (9).

We show here that both hypoxia and etoposide produce an efficient purging of the staminal compartment, synergizing to abolish the malignant N cell production and promote stem cell progression through the benign fibromuscular lineage.

Materials and Methods

Cell culture. The human neuroblastoma SY5Y clone and its derivatives were maintained in RPMI 1640 and cultured as previously reported (2).

Hypoxic conditions (0.1% O₂) were achieved by culturing the cells in an anaerobic workstation incubator (Concept 400, Jouan, Milan, Italy) flushed with a gas mixture containing 0.1% O₂, 5% CO₂, and balanced N₂ at 37°C in a humidified atmosphere (10). Oxygen tension in the medium was measured with a portable trace oxygen analyzer (Dräger PACIII).

The cells were detached from culture plates with a solution of 0.05% trypsin and 0.02% EDTA in PBS (Euroclone, Milano, Italy) and were plated at a density of 10⁵/mL in 24 multiwells. Etoposide (VP-16-213, Sigma, Milano, Italy; dissolved in medium) at 20 μmol/L was added two days after cell seeding, maintained for the indicated times, and removed by substituting the culture medium with a drug-free fresh medium. Untreated (control) cultures were also subjected to change of medium at the same incubation times.

Cell viability was determined by the crystal violet staining method (11).

Immunofluorescence. Immunofluorescence was done essentially as previously described (2).

Each cell population was tested for the following list of antibodies: mouse anti-smooth muscle actin (1:800; Sigma); mouse anti-calponin (1:3,000; Sigma); mouse anti-*nestin* (1:800; BD Transduction Laboratories, Milano, Italy); mouse anti-neurofilament 68 (1:1,500; Sigma); mouse anti-neurofilament 160 (1:2,000; Sigma); mouse anti-neurofilament 200 (1:2,500; Sigma); rabbit anti-p75 (1:800; Chemicon, Milano, Italy); mouse anti-vimentin (1:3,000; Sigma); mouse anti-CD133 (1:1; Miltenyi Biotec, Bologna, Italy); mouse anti-CD117 (c-kit; 1:50; BD PharMingen, Milano, Italy); and mouse anti-microtubule-associated protein 2 (MAP2; 1:2,500; Sigma).

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Patch-clamp recordings. Cells were seeded on 35-mm Petri dishes (Corning, Milano, Italy) and traces recorded with the patch-clamp amplifier MultiClamp 700A (Axon Instruments, Foster City, CA) using the whole-cell configuration or the perforated patch to record the resting potential (V_{REST}) and the action potential (2). The following ion currents (12, 13) were measured during incubation: I_{HERG} , mediated by the Human *eag* related (HERG) potassium channels; I_{KDR} , mediated by the Delayed Rectifier Potassium (KDR) channels; I_{Na} , mediated by sodium tetrodotoxin-sensitive channels; and I_{KIR} , mediated by the Inward Rectifier Potassium (KIR) channels. For a precise measurement of the current gating parameters, pipette, cell capacitance, and series resistance (up to 70–80%) were carefully compensated before each voltage clamp protocol run. The protocol used to measure the tail I_{HERG} maximal current (I_{MAX}) started from a holding of 0 mV and testing the current at -120 mV after preconditioning from 0 to -70 mV for 15 s. The I_{KIR} and I_{HERG} currents were elicited at various potentials (from 0 to -140 mV), starting with a holding potential of 0 or -70 mV, following the practice of Faravelli et al. (ref. 14; currents were obtained at

$[K^+]_0 = 40$ mmol/L). For the activation of I_{KDR} , cells were preconditioned at -80 mV and test potential ranged from -10 to $+70$ mV, with step increments of 10 mV. For activation of tetrodotoxin-sensitive I_{Na} , cells were preconditioned at -90 mV and currents recorded from -40 to $+30$ mV with step increments of 10 mV. This protocol also reveals I_{KDR} , without overlapping of the two currents, which activate quite distantly on the time scale.

Results

The Neuroblastoma Cell Subpopulations Spontaneously Developing in Culture on Removal of N Cell Compartment: Immunocytochemical and Electrophysiologic Phenotypes

The parental clone was nearly 100% constituted of N-type cells positive to neurofilament 68 and MAP2 and expressed the ECDN,

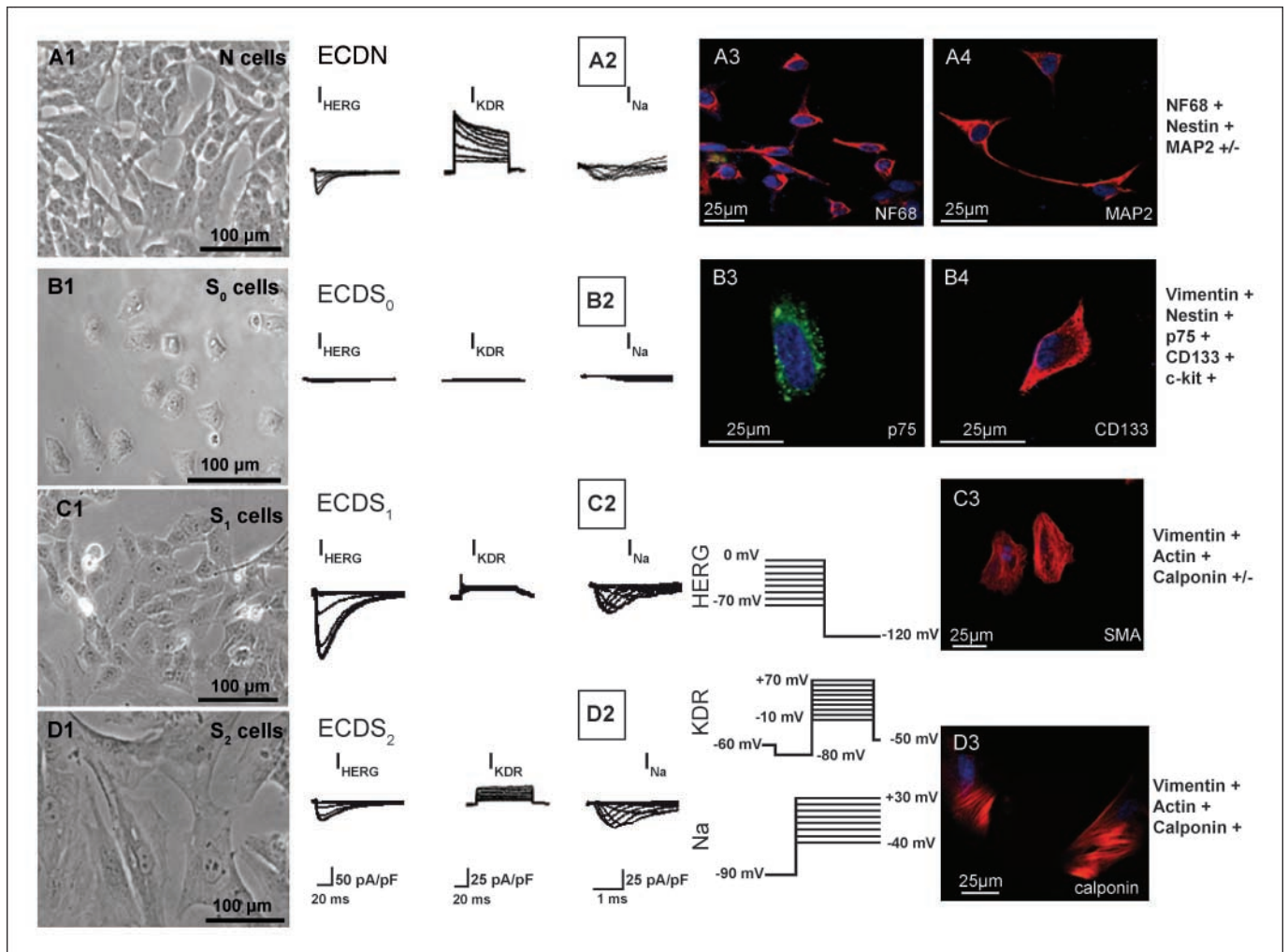


Figure 1. Morphologic, electrophysiologic, and immunocytochemical profiles of various cell types spontaneously developing from the SY5Y clone. A1 to A4, the parental clone is nearly 100% constituted of N-type cells (A1) and expresses the ECDN (substantial I_{HERG} , strong I_{KDR} , and scanty I_{Na} ; A2). The neuronal commitment of these cells is supported by the expression of the immunocytochemical markers neurofilament 68 (NF68) and MAP2 (A3 and A4), whereas their persistent immaturity is indicated by the expression of nestin (see the whole immunocytochemical profile on the last row on the right). Cells were patched in the whole-cell configuration and the current density was expressed in pA/pF. The currents are specifically abolished (not shown) by the following inhibitors with the respective concentrations: I_{HERG} , 2 μ mol/L WAY 123,398; I_{KDR} , 20 mmol/L tetraethylammonium; I_{Na} , 2 μ mol/L tetrodotoxin. B1 to B4, morphology of S_0 population derived by SY5Y clone on limiting dilution (B1). The electrophysiologic profile of these cells (ECDS₀) is characterized by the absence of any substantial current (nude profile; B2), whereas their immunocytochemical profile is distinguished by the expression of the typical neural crest stem cells markers p75 and CD133 (B3 and B4). C1 to C3, after continuously removal of N-type cells, the S_0 component is progressively substituted by S_1 cells (C1). The S_1 electrophysiologic profile (ECDS₁) shows an increase of I_{HERG} and I_{Na} with no substantial change in I_{KDR} (C2). The expression of smooth muscle actin (SMA) supports an early fibromuscular commitment (C3). D1 to D3, after 3 mo, the culture seems to be prevalently populated by S_2 cells. The electrophysiologic profile of these cells (ECDS₂) as compared with ECDS₁, shows a marked increase of I_{HERG} and a substantial increase of I_{KDR} (D2), whereas the expression of calponin (D3) reflects the further cell commitment to the fibromuscular lineage.

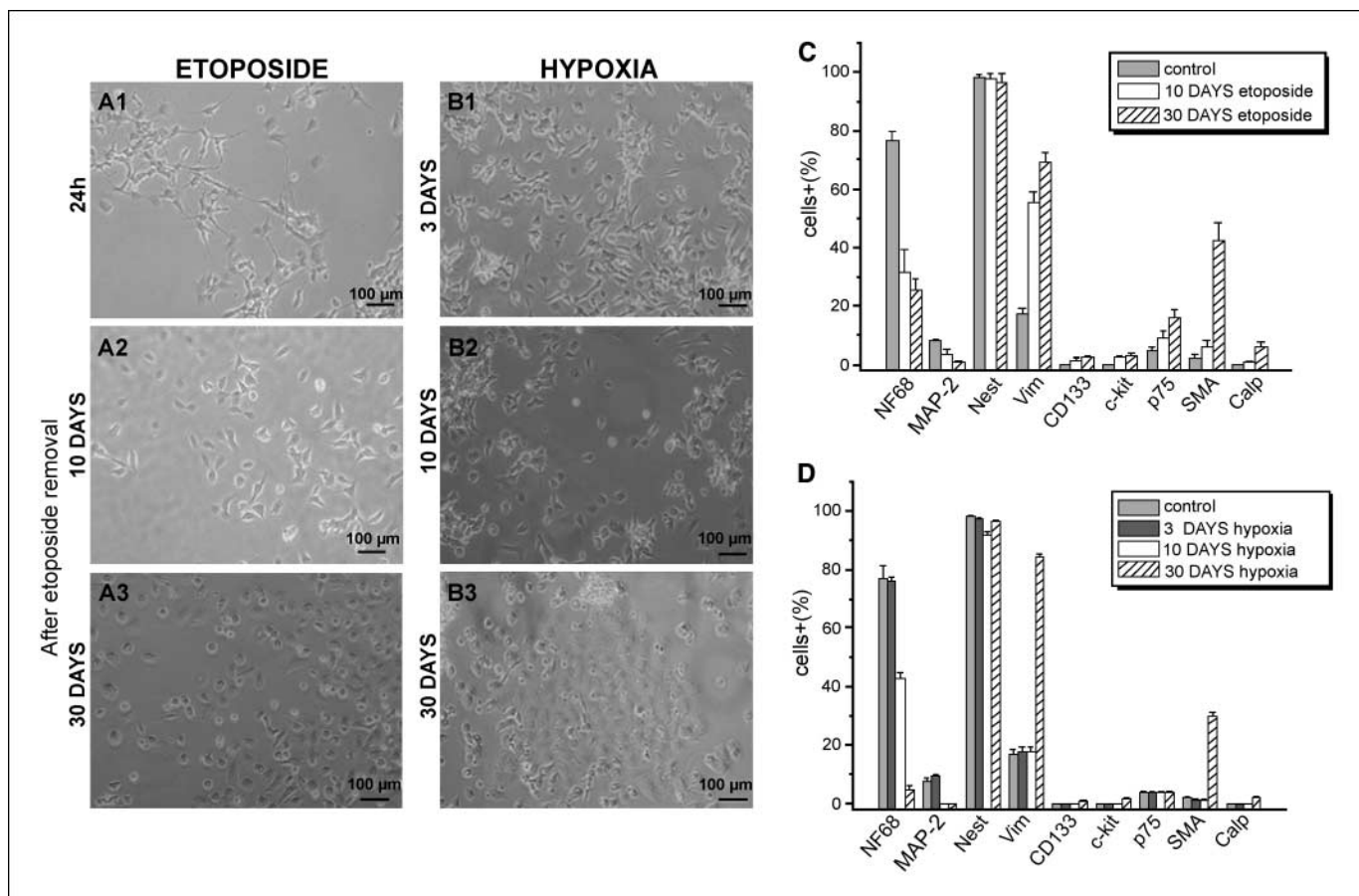


Figure 2. Morphologic and immunocytochemical profiles of neuroblastoma cells after etoposide addition or during hypoxic incubation. *A1* to *A3*, cell cultures soon after etoposide removal or after 10 or 30 d of recovery; *B1* to *B3*, cultures at 3, 10, or 30 d of hypoxic incubation. *C* and *D*, corresponding immunocytochemical profiles during recovery from etoposide or hypoxia. Columns, mean of five experiments; bars, SE.

which was characterized by a substantial I_{HERG} , a strong I_{KDR} , and a scanty I_{Na} (Fig. 1A1–A4). On limiting dilution of the SY5Y population, stem cell (S_0) subclones emerged, which were constituted of epithelial-like strongly adherent cells (Fig. 1B1). For ~15 to 20 days after seeding, the morphology of these subclones remained unvaried and they expressed the “nude” (current devoid) electrophysiologic profile (ECDS₀; Fig. 1B2). Characteristically, S_0 cells expressed the p75, CD133, and c-kit markers (Fig. 1B3 and B4) typical of the neural crest stem cells. Initially, N-type cells were prevalently generated from S_0 (2) but, provided that they are continuously removed, the culture became progressively constituted of fibromuscular committed cells (S_1 – S_2 ; Fig. 1C and D), whose electrophysiologic profiles (ECDS₁ and ECDS₂) were, at first, characterized by the increase of I_{HERG} and I_{Na} (ECDS₁) and then by decline of I_{HERG} and increase of I_{KDR} (ECDS₂; Fig. 1C2 and D2). The S_1 – S_2 progressive fibromuscular commitment was supported by the expression of smooth muscle actin and calponin (Fig. 1C3 and D3). This expression is invariably followed by the exhaustion of the culture, indicating that the S_2 stage represents a terminal differentiation step of the original staminal pool.

Effects of Etoposide and Hypoxia on the Neuroblastoma Cell Fate

Cytokinetics and cell recovery. To study the etoposide effect, we seeded $10^5/\mu\text{L}$ SY5Y cells in the presence of 20 $\mu\text{mol/L}$ drug. Here, the cell population underwent a progressive decrease in cell

numbers as well as in the N/S cell ratio up to the end of the incubation, when the S cells represented the vast majority of the population. The removal of the drug at 24 h determined a substantial recovery due to the fact that, at this time, S cells started proliferating while N cells continued to die. In the long term, this treatment produced a complete disappearance of N cells and the replenishment of the culture with S-type cells. This protocol (20 $\mu\text{mol/L}$ etoposide at time 0, removal of the drug 24 h later, and follow-up to the end of incubation) was applied in the experiments reported below.

Immunofluorescence. Figure 2 reports the morphology and cell markers identified by immunofluorescence at various developmental stages of the neuroblastoma cell population exposed to etoposide for 24 h. Soon after drug removal (Fig. 2A1), the N cells were strongly diminished in number (compare with Fig. 1A1), but S-type cells were still scanty. At day 10 of recovery, the ratio of N/S cells was inverted (Fig. 2A2). The N replacement by S viable cells became nearly complete at day 30 of recovery (Fig. 2A3). This trend was confirmed by the immunocytochemical data reported in Fig. 2C. In fact, within 30 days of recovery, the percentage of cells positive to neurofilament 68 and MAP2 was strongly reduced, whereas the percentage of the following two types of cells was found to be increased: (a) cells identifiable by markers of S_0 cells (CD133, c-kit, and, to a major extent, p75); (b) committed fibromuscular progenies S_1 and S_2 (smooth muscle actin and calponin positive). Such commitment did not exclude the

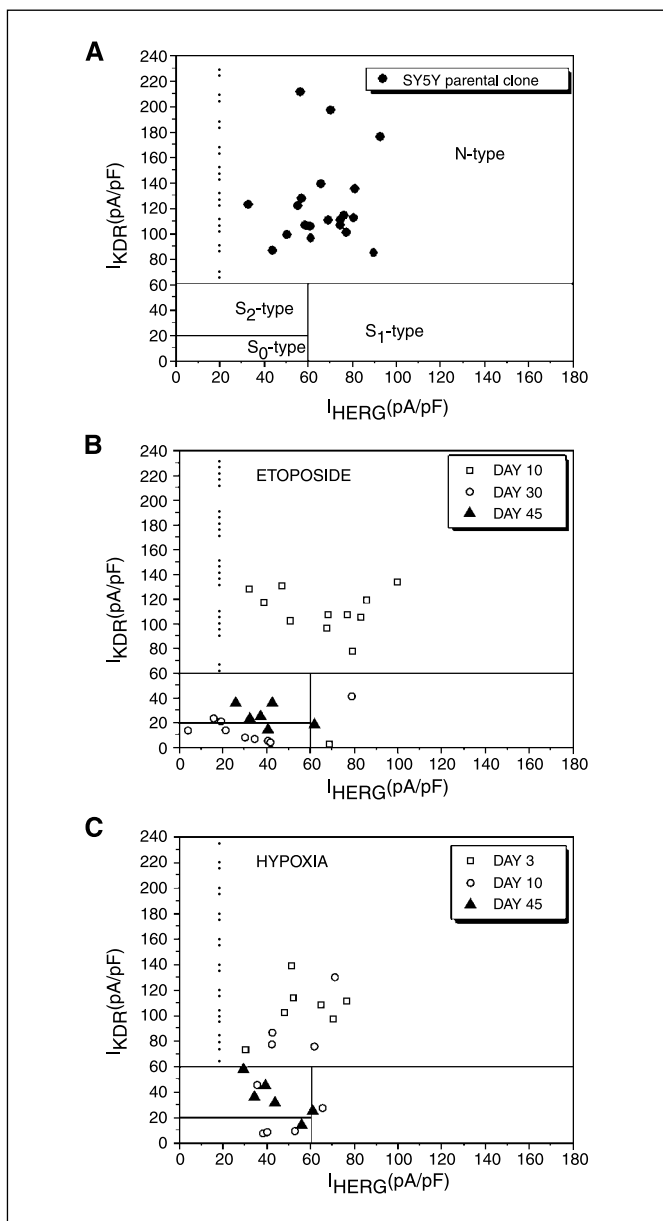


Figure 3. The use of the electrophysiological profiles (ECD) to characterize the neuroblastoma cells: the I_{KDR}/I_{HERG} diagram. A to C, ECD measured in various cell types and treatments represented by the plot I_{KDR} versus I_{HERG} (see text for explanation). Symbols represent currents recorded in each single cell of the SY5Y parental clone (A) at various days after recovery from etoposide (Fig. 3B) or during hypoxia (C). Currents were measured in voltage clamp in the whole-cell configuration. The pipettes used (borosilicate glass, Harvard Apparatus, Kent, United Kingdom) had resistances ranging between 3 and 5 M Ω . Gigaseal resistances were in the of range 1 to 10 G Ω . The cell capacitance was obtained directly by reading the cell capacitance compensation. Input resistances of the cells were in the range of 2 to 6 G Ω . Whole-cell currents were filtered at 1 to 3 kHz. For data acquisition and analysis, the pClamp and Axoscope software (Axon Instruments) and Origin (Microcal Software, Northampton, MA) were routinely used. Extracellular solutions were delivered with hypodermic needles inserted into a capillary with a small hole (inner diameter, 0.4 mm), positioned near the cell under study. The extracellular solution with low potassium (low K_0 solution [K^+] $_0$ = 5 mmol/L) contained (in millimolar) NaCl 130, KCl 5, CaCl $_2$ 2, MgCl $_2$ 2, HEPES-NaOH 10, glucose 5, pH 7.4. The extracellular solution with high potassium (high K_0 solution [K^+] $_0$ = 40 mmol/L) contained (in millimolar) NaCl 95, KCl 40, CaCl $_2$ 2, MgCl $_2$ 2, HEPES-NaOH 10, glucose 5, pH 7.4. The standard pipette solution at [Ca^{2+}] = 10 $^{-7}$ mol/L contained (in millimolar) K $^+$ aspartate 130, NaCl 10, MgCl $_2$ 2, CaCl $_2$ 2, EGTA-KOH 10, HEPES-KOH 10, pH 7.4. For current-clamp experiments, the pipette solution contained (in millimolar) K $^+$ aspartate 140, NaCl 10, MgCl $_2$ 2, HEPES-KOH 10, amphotericin B 150 μ g/mL, pH 7.3.

persistence of a significant degree of immaturity, as indicated by the 100% cell positivity to nestin (15).

Taken in their entirety, these results indicated the following effects of etoposide: (a) the abolition of the N cell compartment and (b) the induction of the S $_0$ -S $_1$ -S $_2$ pattern of differentiation. These results closely mimic the effects of repeated mechanical removal of N cells from the neuroblastoma culture, which were reported in our previous work (2).

Similar conclusions can be drawn from the experiments testing the effect of hypoxia on the neuroblastoma evolution in culture (Fig. 2B1-B3). During the first 3 days of incubation in hypoxia, the cell population did not undergo any significant morphologic or viability change, as compared with the control parental SY5Y clone (see Figs. 1A1 and 2B1). On the other hand, at day 10, the culture already manifested a marked inversion of the N/S ratio (Fig. 2B2).

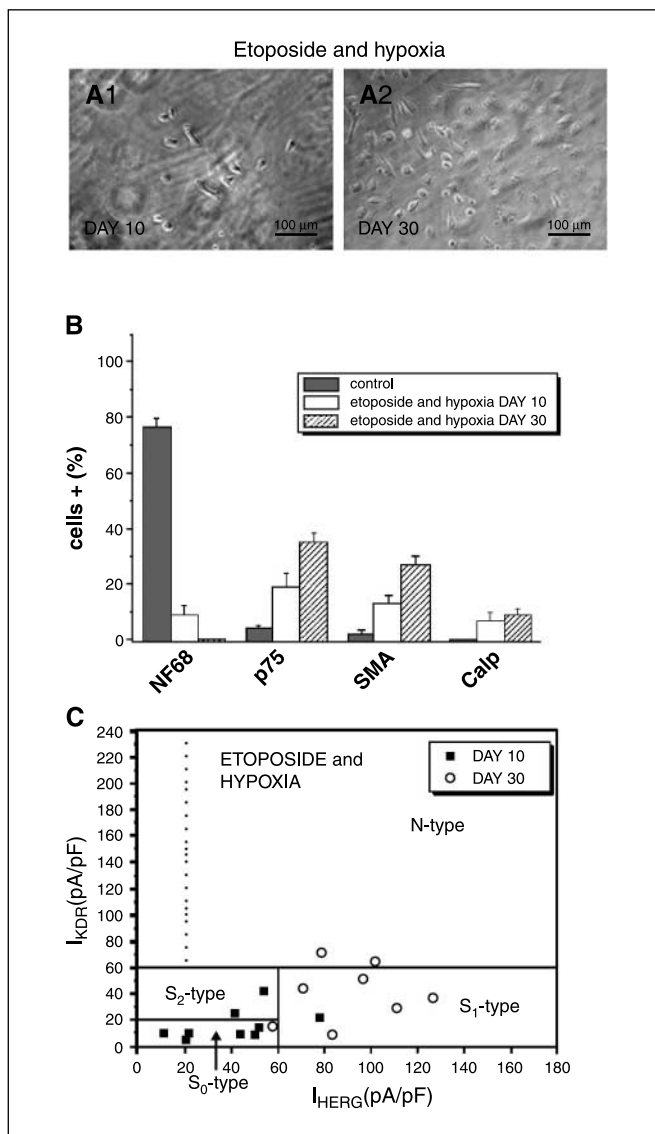


Figure 4. The combined etoposide and hypoxic treatment. Cells were exposed to hypoxia and etoposide at time 0. The drug was removed after 24 h, whereas hypoxia was maintained for 10 or 30 d. A1 and A2, cell morphology. B, cell immunocytochemical profile. Columns, mean of four experiments; bars, SE. C, electrophysiological profile (ECD) estimated as in Fig. 3.

This effect of hypoxia increased with time, so that at day 30 the cell population was almost exclusively constituted of living S-type cells (Fig. 2B3). Thus, hypoxia affects the neuroblastoma evolution in a very similar way as etoposide, although at a faster rate. The same conclusion applies to the expression of the prevalent cell marker profile, which, within 30 days, shifts from the N-type cells (neurofilament 68 and MAP2 positive) to those expressed during the S₀-S₁-S₂ progression (smooth muscle actin and calponin positive; Fig. 2D).

ECD. The time course of ECD during the neuroblastoma population development in the absence of or after treatment with etoposide or hypoxia is illustrated in Fig. 3. In this figure, patch-clamp data are represented using the plot I_{KDR} versus I_{HERG} , which proved particularly suitable to discriminate cell subpopulations on the basis of the two major currents simultaneously expressed in each single cell (2). In the case of the SY5Y clone and its derivatives, this approach allowed the identification of four compartments (Fig. 3), including one or other of the cell types composing the entire population. The limits of these compartments were traced as follows: N cell compartment, $I_{HERG} > 20$ pA/pF, $I_{KDR} > 60$ pA/pF; S₀ compartment, $I_{HERG} < 60$ pA/pF, $I_{KDR} < 20$ pA/pF; S₁ compartment, $I_{HERG} > 60$ pA/pF, $I_{KDR} < 60$ pA/pF; S₂ compartment, $I_{HERG} < 60$ pA/pF, 20 pA/pF $< I_{KDR} < 60$ pA/pF.

As shown in Fig. 3A, the totality of the SY5Y parental cells seemed to be included in the N compartment, in keeping with the large prevalence of this population in this clone. On the other hand, at 30 days of recovery from etoposide (Fig. 3B), the N compartment became empty while cells were accumulated in the S₀, S₁, and S₂ compartments. The same considerations apply to the effects of hypoxic incubation, in particular at day 45 (Fig. 3C).

On the whole, the electrophysiologic screening is consistent with the immunocytochemical analysis of the etoposide and hypoxic treatments illustrated above.

In conclusion, the overall neuroblastoma cell population responds to both etoposide and hypoxia with a profound modification of its composition. The N subset, which dominates in the parental clone, is indeed progressively abolished, leaving space for the onset and development of the S subsets, up to the absolute predominance of the S₂ cells at the end of culture.

The Synergy of Etoposide and Hypoxia Treatments

The similarity of effects displayed by etoposide and hypoxia was further explored by testing the combination of the two treatments. At time 0, cells were simultaneously exposed to hypoxia and etoposide. The drug was removed after 24 h, whereas hypoxia was maintained throughout the experimental time. Morphologic, immunocytochemical, and electrophysiologic data (Fig. 4A1-C) concurred to indicate that, as compared with the separate treatments, the combined treatment produces (a) a marked acceleration of the N cell compartment abolition (compare Figs. 2-4) and (b) a more rapid development of the S₀, S₁, and S₂ components.

Discussion

There are two major facts to emerge from this study. First, the cell reservoir represented by the S₀ staminal compartment is substantially insensitive to apoptotic stimuli, and it transmits this insensitivity to progeny of the S-type but not the N-type. Second, hypoxia and etoposide affect the spontaneous development of SY5Y clone in the same way, leading initially to the progressive

elimination of the N cell compartment and thereafter to the purging of the S₀ cells and their terminal commitment to the fibromuscular phenotype.

All in all, our study suggests that hypoxia, which we have shown to allow the purging of the neuroblastoma staminal compartment *in vitro*, might have important implications for the natural history of this tumor *in vivo*. In fact, neuroblastoma, just like any other malignancy, unavoidably develops ischemic areas, selecting hypoxia-resistant clones (16, 17). In these areas, the peculiar effect of hypoxia described in this article may manifest itself with the abolition of the aggressive N component and with the S₀ terminal commitment to the benign fibromuscular phenotype.

A major question still to be resolved concerns the status of S₀ cells in the context of the neuroblastoma population. The established features of these cells are the following: (a) a high clonogenic potency (we followed a single-cell clonal expansion up to hundreds of cells); (b) an at least bipotent plasticity (neuronal and fibromuscular); (c) the requirement of an abundant N cell progeny to maintain the S₀ self-renewal capacity (2); (d) the abolition of the S₀ option for the N lineage when N cell generation is impaired; and (e) the exhaustion of the S₀ staminal status in hypoxia.

As far as we know, no example of similar behavior in normal or cancer stem cells has been described thus far. We are inclined to trace this behavior back to the tendency of the neural crest staminal compartments to generate more or less committed progenitors, which nevertheless maintain a substantial capacity for self-renewal together with clonogenic potential (18). It is conceivable that S₀ cells belong to a progenitor category of this kind, restricted to the neuronal and fibromuscular lineages. Of the two possible fates, the neuronal differentiation pathway is evidently arrested at a defective stage, driving the overwhelming proliferation of the committed N progenitors and monopolizing the S₀ progeny before its bifurcation. The N cell population might contribute to the maintenance of the staminal status of S₀ cells with soluble factors or cell-to-cell contacts. Only when this contribution is abolished by mechanically removing the N cells or by hypoxic or antiproliferative treatment can the S₀ cells fully accomplish their terminal differentiation. This interpretation of the evidence implies that neoplastic transformation in the SY5Y clone reflects a differentiation block downstream from the stem cell compartment.

In conclusion, the S₀ cell behavior that emerges from our study is the first experimental hint to explain the reported spontaneous or inducible regression of this tumor from a malignant to a benign phenotype (7, 19). Indeed, the response of S₀ cells to hypoxia seems to be able to sustain this conversion, promoting the differentiation of stem cells instead of freezing them in a dormant state, as what happens in most types of tumor (1, 20). At the same time, the synergy of hypoxia with etoposide, far from being an empirical abstraction, might have an important role to play in the context of ischemic areas of tumors treated with antiproliferative drugs.

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