Effect of Microtubule Disorganizing or Overstabilizing Drugs on the Proliferation of Rat 3T3 Cells and Their Virally Induced Transformed Derivatives

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ABSTRACT

Drugs that disorganize or overstabilize cytoplasmic microtubules (colchicine, vinblastine, griseofulvin, or taxol) can at certain concentrations totally block proliferation of SV40 and polyoma virus transformants with only a minimal effect on the proliferation of the parental rat 3T3 cells. This difference in sensitivity is not due to a more active drug uptake by transformed cells. Examination of cytoplasmic microtubules in actively proliferating normal or transformed cells reveals two categories in each case: cells with microtubules and cells without distinct microtubules. The proportion of cells without distinct microtubules did not differ much between normal and transformed cells. However, transformed cells with a clear microtubule network appear to have fewer microtubules than normal cells. This may contribute to the higher sensitivity of transformed cells. These results render even more rational the use of antimicrotubule drugs in cancer chemotherapy.

INTRODUCTION

Microtubules, intermediate filaments, and actin microfilaments are the primary components of the cytoskeleton in mammalian fibroblasts grown in culture. They are networks of cytoplasmic fibers that can be visualized by indirect immunofluorescence using antibodies directed against the protein monomer that constitutes the fiber.

Microfilaments are clearly implicated in the processes that ensure spreading and adhesion of the cell to the substratum and in certain cell surface movements, namely the development of lamellas and ruffling (1, 2). They also have a role in cell translocation and in the mobility of surface receptors (3–9). Microtubules also play crucial roles in fundamental cell functions, namely determination and maintenance of cell shape and intracellular architecture, transport of vesicles, cell translocation, and surface movements like extension and retraction of processes, blebbing, and mobility of surface receptors. They are also essential elements of the mitotic spindle (5–8, 10–14).

Microtubules may also have an additional major role; they appear to be implicated in the control of cell proliferation and in the mechanisms of oncogenic cell transformation. Indeed, a transient depolymerization of microtubules by drugs that promote microtubule disassembly is sufficient to induce DNA synthesis and division in resting cells cultivated without serum (15). Numerous reports had previously indicated that in the presence of serum, microtubule depolymerization induces DNA synthesis in resting cells or enhances the effect of various mitogens (3, 16–20). Furthermore, it was shown that in vivo stabilization of microtubules by taxol inhibits the mitogenic effect of epidermal growth factor and thrombin on resting cells (21). Known tumor promoters like griseofulvin (22) disrupt microtubules both in vivo and in vitro (23), and drugs like vinblastine, which prevent tubulin polymerization into microtubules, increase, like tumor promoters, the frequency of cell transformation induced by polyoma virus (24).

The network of microtubules was reported to be altered in some transformed cells (25). The correlation, however, between transformation and alteration of the microtubule network has not been endorsed by other investigators (26–29). It was argued that the differences in microtubule organization between normal and transformed cells are only apparent and stem from changes in the shape and degree of spreading of transformed cells. This fact renders difficult the documentation of the microtubule network.

We have examined the effect of drugs that disorganize or stabilize microtubules on the proliferation of rat 3T3 cells and on their transformed derivatives induced by polyoma virus or SV40. The SV40 transformants used in this study display all the growth properties typical of maximal transformants but cannot be distinguished easily from the parental 3T3 cells on morphological grounds or on the basis of growth rate. They maintain the typical fibroblastic elongated and bipolar shape and are neither smaller nor more retracted than the parental cells. The polyoma virus transformants used in this study are more "progressed" in the pathway of transformation than the SV40 transformants. They proliferate faster under conditions restrictive for normal cell proliferation and have more profound membrane alterations (30, 31).

MATERIALS AND METHODS

Cells, Medium, Chemicals, and Antibodies. The rat 3T3 cells and their derivatives transformed by polyoma virus or SV40 were all described previously (30, 31). They were grown at 33°C in Dulbecco's Modified Eagle medium supplemented with 10% newborn calf serum. Vinblastine (M, 909) and colchicine (M, 399) were purchased from Sigma. Griseofulvin (M, 352) was from Aldrich and taxol (M, 820) was a gift from Dr. Daniel Guenard, Institut de Chimie des Substances Naturelles, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France. Stock solutions were all in dimethyl sulfoxide. Pure anti-tubulin sheep antibodies were prepared as described previously (32) and Texas red conjugated goat IgGs directed against sheep IgGs were from Amersham. Tritium labeled vinblastine (10 Ci/mmol) was also from Amersham.

Cell Proliferation. Actively proliferating normal or transformed cells (10⁶ cells/25 cm²) were treated with the drug at the indicated concentrations; control cells were treated with dimethyl sulfoxide (1/1000 dilution). The extent of cell proliferation was determined by calculating the logarithm of the ratio N₃/N₀, the number of cells after 3 days of incubation in the presence of the drug over the number of cells on the day the drug was added.

Uptake of Labeled Vinblastine. Actively proliferating normal or transformed cells (10⁶ cells/25 cm²) were incubated at 33°C from 0 to 180 min with 25 × 10⁻⁸ M tritium labeled vinblastine. At the end of the incubation period, the cells in the flask were washed four times with 10 ml PBS (phosphate buffered saline) and lysed with 2 ml 1 N sodium hydroxide and the lysate was incubated for 30 min at 60°C. A 200-μl aliquot was used to determine the total protein content and the rest was used to determine the total radioactivity retained by the cells. All points were in triplicate.

Indirect Immunofluorescence. The cells were fixed in 3.7% formaldehyde for 30 min, permeabilized with 0.1% Triton for 5 min, and incubated with sheep anti-tubulin antibodies for 30 min and then with goat anti-sheep antibodies for 30 min. After extensive washing the cytoplasmic microtubules were viewed by epifluorescence using a Zeiss Universal microscope.

Received 4/7/86; revised 7/15/86, 3/21/88; accepted 6/1/88.

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RESULTS

Effect of Microtubule Disrupting Agents on Cell Proliferation. Proliferating rat 3T3 cells, SV40 transformants (SV3T3) or polyoma virus transformants (PY3T3) were treated with various doses of vinblastine (5 to 50 × 10⁻⁸ M), colchicine (1 to 6 × 10⁻⁷ M), or griseofulvin (5 to 50 × 10⁻⁶ M) and their proliferation was assessed 3 days later (Fig. 1). In the case of SV40 or polyoma virus transformants a 50% inhibition of proliferation was observed at 7.5 × 10⁻⁸ M vinblastine, 1.5 × 10⁻⁷ M colchicine, and 12.5 × 10⁻⁶ M griseofulvin. In the case of normal cells a 50% inhibition of proliferation required higher doses: 27.5 × 10⁻⁸ M vinblastine; 2.5 × 10⁻⁷ M colchicine; and 30 × 10⁻⁶ M griseofulvin. Moreover, proliferation of SV40 or polyoma virus transformants was totally blocked at 15 × 10⁻⁸ M vinblastine, 3 × 10⁻⁷ M colchicine, and 15 × 10⁻⁶ M griseofulvin. At those doses the normal cells could still proliferate well during 7 additional days; the medium containing the drug was renewed every 3 days. Normal cell proliferation was blocked only with 50 × 10⁻⁸ M vinblastine; 7 × 10⁻⁷ M colchicine; and 40 × 10⁻⁶ M griseofulvin.

Six additional transformed cell lines induced either by polyoma virus or SV40 (30) were assayed for their ability to proliferate in the presence of microtubule disrupting agents. None could proliferate in the presence of 15 × 10⁻⁸ M vinblastine, 3 × 10⁻⁷ M colchicine, or 15 × 10⁻⁶ M griseofulvin. Thus transformants derived from rat 3T3 appear to be more sensitive than their parental cells to the inhibitory effect on proliferation of vinblastine, colchicine, or griseofulvin. All three agents act directly on tubulin and interfere with the autopolymerization process that generates microtubules (11).

Effect of Taxol on Cell Proliferation. Taxol is an agent that overstabilizes microtubules (33, 34). It considerably diminishes the critical concentration of free tubulin monomer required for polymerization (35). Proliferating normal or transformed cells were treated with taxol at doses ranging from 1 to 10 × 10⁻⁷ M (Fig. 2). A 50% inhibition of proliferation required a dose of 3 × 10⁻⁷ M for the transformed cells and a dose of 5 × 10⁻⁷ M for normal cells. Total inhibition of transformed cells proliferating normal or SV40 transformed cells. Fig. 3 shows that normal and transformed cells transport vinblastine at a similar rate and accumulate this drug to a similar level. Differences in sensitivity towards vinblastine do not therefore come from an increased poisoning of transformed cells due to a more active transport.

Radiolabeled vinblastine was therefore added to a final concentration of 25 × 10⁻⁸ M to the medium of actively proliferating normal or SV40 transformed cells. Fig. 3 shows that normal and transformed cells transport vinblastine at a similar rate and accumulate this drug to a similar level. Differences in sensitivity towards vinblastine do not therefore come from an increased poisoning of transformed cells due to a more active transport.

Morphology and Microtubules in Proliferating Normal and Transformed Cells. The normal rat 3T3 cells used in this study are regular fibroblasts which when sparse are mostly elongated, bipolar, and flat (Fig. 4A). The SV40 induced transformed derivatives do not differ much from their parental cells, they remain mostly elongated and bipolar with only narrower cell extensions and are well spread on the substrate (Fig. 4B). On the other hand, the polyoma virus induced transformants are multipolar but are still flat and well spread on the substrate (Fig. 4C). Previous reports had indicated that the microtubule networks are less well organized in transformed cells. This view, however, was seriously challenged by other investigators on the grounds that the differences were only apparent and resulted from the fact that transformed cells have less well spread cytoplasm (28).
Fig. 3. Uptake of tritium labeled vinblastine by normal (A) and SV40 transformed cells (SV3T3) (B). Actively proliferating cells (10⁶/25 cm²) were incubated with ³H labeled vinblastine. Error bars vary from 4 to 16%.

or SV40 transformed cells (Fig. 5). Two categories could be clearly distinguished among normal cells (Fig. 5; Table 1): cells with numerous microtubules; and cells without distinct microtubules with an intense diffuse background of free tubulin. The same results were obtained using the procedure described by Osborn and Weber (28) where cell fixation and extraction are made in a microtubule stabilizing buffer.

In the case of SV40 transformants, two categories were also clearly distinguished (Fig. 5; Table 1): cells with microtubules; and cells without distinct microtubules. The proportion of cells with microtubules was similar in normal and transformed cells. There is a slight shift in transformed cell population toward cells without distinct microtubules. The immunofluorescent visualization of these cells also suggested that each transformed cell that had distinct microtubules appeared to have fewer microtubules than normal cells.

Microtubules in Resting Cells. When the cells were allowed to reach confluence in 10% serum or were incubated in 1% serum for 2 days while still isolated, the great majority (>95%) were found to have a well developed microtubule network (Fig. 6; Table 1). This strongly argues (a) against the presence of two distinct clonable populations and (b) against the possibility of a fixation artifact. The data, however, appear to favor the possibility that microtubules are massively disorganized during a certain period of the cell cycle which lasts longer than the immediate premitosis phase.

DISCUSSION

This paper reports that drugs which disorganize or stabilize microtubules can at certain concentrations totally block proliferation of transformed cells with only a minimal effect on normal cell proliferation. This difference is not due to a more active uptake of these drugs by transformed cells. Examination of cytoplasmic microtubules in actively proliferating normal and transformed cells shows that the transformed cells do have microtubules arranged in networks, but the number of microtubules per cell is reduced compared to normal cells. The reduced number of microtubules per cell may contribute to the higher sensitivity of transformed cells to microtubule disrupting or overstabilizing drugs.

Previous reports had indicated that the number, the organization, and the integrity of microtubules are different in transformed cells (25). The validity of these findings was later doubted. The following arguments were raised (28). When large and well spread normal cells are compared to small and re-
DISORGANIZING-OVERSTABILIZING DRUGS AND CELL PROLIFERATION

Fig. 5. Cytoplasmic microtubules in proliferating normal or SV40 transformed cells. A, B, normal cells; C, D, SV40 transformed cells. Bar, 15 μm.

Table 1 Cytoplasmic microtubules in actively proliferating normal, SV40 transformed, and quiescent rat 3T3 cells

<table>
<thead>
<tr>
<th>Cells</th>
<th>Cells with distinct microtubules (%)</th>
<th>Cells without distinct microtubules (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferating 3T3</td>
<td>50</td>
<td>46</td>
</tr>
<tr>
<td>Proliferating SV3T3</td>
<td>42*</td>
<td>61</td>
</tr>
<tr>
<td>Quiescent 3T3</td>
<td>&gt;95</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

* Each transformed cell had fewer microtubules than a normal cell (Fig. 5, A and C).

When treated with limited amounts of vinblastine that affect only slightly their proliferation.

When the normal rat 3T3 cells are treated with limited amounts of vinblastine that affect only slightly their prolifera-
Cells are not affected. In this paper it is shown that even proliferation normal cells are less sensitive than their transformed derivatives to agents that alter normal tubulin polymicrotubule drugs is sufficient to induce DNA replication in microtubules in 3T3 cells after attachment to a substratum. Exp. Cell Res., 102: 321-340, 1976.

Drugs that disorganize microtubules are used in cancer chemotherapy. The reasoning behind their use is the following. By disrupting microtubules that are an essential part of the mitotic spindle the proliferating cancer cells in a tissue are prevented from dividing, while the surrounding resting normal cells are not affected. While proliferating normal cells are less sensitive than their transformed derivatives to agents that alter normal tubulin polymerization.

The results reported in this paper are consistent with the hypothesis that microtubules play a central role in cell proliferation. Indeed disruption of cytoplasmic microtubules by antimicrotubule drugs is sufficient to induce DNA replication in resting cells (5, 15, 21). The fact that both disrupting and stabilizing agents have the same biological effect (this paper) indicates that a certain flexibility of the microtubule network (general or localized cycles of assembly and disassembly) is required for its physiological role.

The cells described in this paper may be used to rapidly assay the toxicity and the in vitro antitumor activity of new drugs that interact with microtubules.

REFERENCES

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