Recruitment of Noncycling Tumor Cells into Proliferation by Isoproterenol

Brad W. Greider, Robert F. Kallman, and Allan J. Franko

Department of Radiology, Stanford University School of Medicine, Stanford, California 94305

ABSTRACT

The resistance of tumors to conventional anticancer therapy is partially determined by the fraction of noncycling cells they contain. In several normal tissues, isoproterenol can stimulate cell proliferation. The effect of isoproterenol on the EMT6/St tumor grown both intradermally in BALB/c mice and in vitro as multicell spheroids was investigated. The incorporation of [3H]thymidine (5 μCi/g, 6.7 Ci/mmol) into DNA was determined as a function of time after i.p. injection of isoproterenol (0.15 mg/g). Tissue section autoradiographs of the EMT6 tumors were prepared, and labeling indices were determined. EMT6 spheroids were exposed to isoproterenol and labeled with [3H]thymidine in a manner designed to simulate drug exposure in vivo. In EMT6 tumors, the labeling index rose from a control level of 30% to a peak of 53% at 25 hr; it then declined gradually for the next 30 hr.

This may be interpreted as evidence that cells were recruited into proliferation. In contrast, isoproterenol had minimal effects on EMT6 spheroids, implying that recruitment caused by this drug was not a direct effect on individual cells.

INTRODUCTION

It is reasonable to expect that tumor cell proliferation kinetics plays a significant role in determining the effectiveness of conventional radiotherapy and chemotherapy. Some cells of a solid tumor are traversing the cell cycle rapidly, others are cycling slowly or are reversibly arrested, and some are irreversibly arrested and destined to die. The former 2 compartments are of clinical significance. Conventional radiotherapy and chemotherapy are most effective at killing rapidly cycling cells, designated for convenience as P-cells, whereas their slowly cycling or reversibly arrested counterparts, designated as Q-cells, may be expected to be relatively therapy resistant. Several models have been developed describing tumor cell proliferation kinetics in terms of 2 compartments and the transit of cells between these 2 compartments (5, 10, 13). Further, Barendsen et al. (1) and Kallman et al. (10) have convincingly demonstrated the clonogenic potential of Q-cells. This redistribution of Q-cells into the proliferating compartment is termed "recruitment" and, in some systems, can theoretically be held responsible for much of the failure of conventional anticancer therapy.

Having established that Q-cells are potentially clonogenic, it is essential to obtain a better understanding of the factors that can cause the conversion of Q-cells to P-cells in vivo, i.e., the factors that cause recruitment. The effectiveness of conventional radiotherapy and chemotherapy could be improved if we were able to increase the fraction of therapy-sensitive cells in a tumor, i.e., recruit Q-cells to P-cells, and then treat with conventional modalities. Barka (2, 3) and Baserga (4) have shown that IPR stimulates DNA synthesis and cellular proliferation in the parotid and submandibular glands of the rat and mouse. In this study, we have investigated the effect of IPR on cellular proliferation in the EMT6/St tumor in BALB/c mice and in EMT6/St spheroids in vitro.

MATERIALS AND METHODS

BALB/cKa mice, produced in our specific-pathogen-free colony, were used at 3 to 5 months of age. EMT6/St tumors (12) were propagated from intradermal inocula of 2 x 10⁵ cells, and when the tumors had grown to 7 to 8 mm in diameter (mean volume, approximately 220 cu mm), mice were given injections of IPR. The drug was freshly dissolved in bacteriostatic normal 0.9% NaCl solution (McGaw Laboratories; 9 mg each of NaCl and benzyl alcohol per ml) and administered i.p. at a dosage of 0.15 mg per g; control animals were given injections of an equal volume of bacteriostatic normal 0.9% NaCl solution. One hr prior to sacrifice, the mice were given injections of [3H]dThd (5 μCi/g, 6.7 Ci/mmol) i.p. At sacrifice, the tumors and submandibular glands were excised, and tissue section autoradiographs were prepared by dipping slides in Kodak NTB emulsion and exposing them for 5 weeks. The LI of tissue section autoradiographs was obtained by examining randomly chosen fields and scoring all cells seen within each field for the absence or presence of more than 5 grains over the nucleus. The LI was defined as the number of nucleated cells with grains per total number of nucleated cells seen. Since the [3H]dThd is incorporated only into the DNA of cells undergoing DNA synthesis at the time of administration and for perhaps 15 to 30 min after it, the LI is approximately equal to the fraction of cycling cells in S phase and is also proportional to the percentage of P-cells. Confidence limits for the data were determined from a standard table of binomial confidence limits (7).

EMT6/St spheroids were initiated by inoculating 2.5 x 10⁴ cells in 60-mm non-tissue culture Petri dishes containing Waymouth's medium with 15% fetal calf serum. Five days later, aggregates roughly 100 μm in diameter had formed. These were transferred to standard spinner cultures (14) containing Waymouth's medium with 15% fetal calf serum, which was replenished daily. After 12 days in spinner culture, the spheroids had grown to diameters of 700 μm, at which time they were divided into 2 groups. One group was maintained as controls (no drug treatment), and the other was exposed to IPR (0.3 mg/ml for 8 hr) to simulate the treatment of tumors. Fresh medium with IPR was given at the midpoint of the exposure because a preliminary experiment had shown evidence of severe toxicity concomitant with a change in the color of the medium to dark brown, and this could be avoided by providing fresh medium with IPR every 4 hr. The spheroids were then grown in medium without IPR for an additional 5 hr. Finally, both groups were labeled for 24 hr in medium containing 0.1 μCi [3H]dThd per ml (6.7 Ci/mmol). A temperature of 37°C was maintained throughout. Subsequent

1 This work was supported by Grants CA03353 and CA10372 from the NIH, Department of Health, Education, and Welfare.2 Present address: Department of Ophthalmology, Veterans Administration Hospital, Sepulveda, Calif. 91343.3 To whom requests for reprints should be addressed.4 Present address: Radiobiology, Cross Cancer Institute, 11560 University Avenue, Edmonton, Alberta, Canada T6E 1W5.5 The abbreviations used are: IPR, isoproterenol; dThd, thymidine; LI, labeling index.
RESULTS

EMT6/St Tumor. The initial LI of the tumors from animals given IPR injections was 29%. This rose to a peak, reaching 53% at 25 hr after injection of IPR (Chart 1). The LI of the control group receiving bacteriostatic 0.9% NaCl solution remained at 29% throughout this period.

Submandibular Gland. Consistent with Barka’s earlier finding (2, 3), the LI of this gland remained near the control level (approximately 2%) until 13 hr after IPR administration. It started to rise at 16 hr, the rate of increase turned sharply upwards after 28 hr, and it reached a peak of about 29% at 32 hr (data not shown). The LI of the control group receiving bacteriostatic normal 0.9% NaCl solution remained at 2% throughout.

Spheroids. The LIs of the cells in spheroids as a function of radial depth for control and IPR-treated spheroids are shown in Chart 2. The decrease in the LI with distance from the spheroid surface is similar to that observed by others using EMT6/Ro spheroids (6). The LIs of individual grid squares were found to be normally distributed within the squares constituting Shells 3 and 4. There was no significant difference in these shells between the LI of control and IPR-treated spheroids, as evaluated by Student’s t test. A subsequent experiment in which medium with IPR was renewed hourly yielded similar results (data not shown). In this experiment, the plating efficiencies of the cells (percentage of cells which form colonies) in IPR-treated and control spheroids were found to be 56 and 60%, respectively. These are slightly higher than the values reported by Freyer and Sutherland (6), but the differences are not significant.

DISCUSSION

The effect of IPR on the EMT6/St tumor is similar to the effects shown by Barka (2, 3) and Baserga (4) on the submandibular gland. The rise in the pulse LI of tumors to 53% may indicate that virtually all of the cells in the tumor were cycling between 22 and 30 hr after injection of IPR. Since the mean cell cycle time of the EMT6 tumor is approximately 22 hr and the mean duration of S phase is approximately 11.8 hr (12), this would predict that the LI for a pulse labeling should be 54% if all cells were in cycle. This is based on the assumption that there was not an appreciable perturbation of the cell cycle kinetics. It is conceivable that the rise in the LI of the tumors may have been caused by prolongation of the S phase, but we have no evidence to support this possibility. The finding that the pulse LI was 53% from 24 to 28 hr after injection of IPR therefore suggests that all tumor cells were in cycle at this time.

The experiment with spheroids was designed to test the possibility that the apparent recruitment of quiescent tumor cells into proliferation was caused by a direct effect of IPR on individual tumor cells. The spheroid is an appropriate model for this experiment because it possesses many of the relevant structural features of the tumor cell.
features of tumors (6, 14) without the added complexity of IPR-induced changes in the host which might also cause recruitment. The spheroid contains rapidly proliferating cells at the surface, presumably equivalent to cells near blood vessels in tumors, and slowly or noncycling cells at the edge of a necrotic center, presumably equivalent to cells far from blood vessels in tumors. Since the data from IPR-treated tumors appear to indicate that cells from all regions of EMT6 tumors were recruited into cycle, a direct effect of IPR was expected to cause a dramatic increase in the proportion of internal cells in spheroids which take up [3H]dThd during continuous labeling. This was found (Chart 2). The small difference found is not significant; indeed, the difference was reversed in the subsequent experiment in which the medium was renewed hourly (data not shown). The 24-hr labeling period is roughly 1.5 times the cell cycle duration of exponentially growing EMT6 cells in monolayer culture and 1.2 times the mean cell cycle duration of EMT6 cells in tumors. The fact that the LI was greater than 90% in the outer 32 μm of the spheroids indicates that the growth fraction is almost 1.0 in this region, so little recruitment could be expected there. Thus, it appears that IPR cannot cause recruitment of quiescent EMT6 cells in the absence of a host, which implies that the recruitment observed in tumors is the result of some host-IPR interaction which subsequently affects the tumor.

ACKNOWLEDGMENTS

We would like to thank Paul Harris and Diane Rapacchietta for their expert technical assistance and Dr. J. Raleigh of the Cross Cancer Institute, Edmonton, Alberta, Canada, for performing the analysis of the rate of breakdown of IPR in growth medium.

REFERENCES

Recruitment of Noncycling Tumor Cells into Proliferation by Isoproterenol

Brad W. Greider, Robert F. Kallman and Allan J. Franko