Progression to Steroid Autonomy in S115 Mouse Mammary Tumor Cells: Role of DNA Methylation

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ABSTRACT Although monoclonal in origin, mammary tumors acquire a marked heterogeneity of cell phenotypes, including a mixture of steroid hormone-sensitive cells and insensitive cells. We describe here long-term studies on the effects of androgen withdrawal on cloned androgen-responsive S115 mouse mammary tumor cells as a model system to investigate mechanisms by which tumor cells lose their steroid sensitivity. In the prolonged absence of androgen, the cells lost hormone-sensitive parameters reproducibly, including loss of proliferative response, saturation density response, cell morphology response, and mouse mammary tumor virus long terminal repeat (MMTV-LTR)-related RNA. These experiments have demonstrated that when deprived of hormone in the long term, a clone of responsive cells gives rise reproducibly to a population of unresponsive cells in an ordered series of phenotypic changes. At the time when the cells lost all androgen response in terms of cell biology and MMTV-LTR-RNA, increased methylation of MMTV-LTR sequences in the DNA was detected. Thereafter recovery of androgen sensitivity has not been achieved in any of these parameters. The possible role of de novo DNA methylation in the progression to androgen autonomy of S115 cells is discussed.

Manipulation of the steroid environment affects breast tumor growth in many species (26, 29) but only on a temporary basis, and even in rodents, where effects are greatest, it never eliminates the tumor completely (19). In man, 30% of breast cancers regress after endocrine therapy, and a recent approach has been to select likely hormone-sensitive tumors on the basis of estrogen and progesterone receptor levels (9). However, possession of receptors is not the sole criterion for response to therapy and in any case regression is at best temporary, to be followed by hormone-independent tumors and metastatic disease. This progression to hormone insensitivity is a major clinical problem.

Tumors are composed of mixed populations of cells that have a wide range of phenotypes. Cells obtained from individual rodent mammary tumors differ not only in hormone receptors (35) and hormone-dependent growth (8, 39) but also in immunological properties (24), transplantability (36), metastatic capability (18), tumorigenicity (38), drug resistance (23), growth rate (11), karyotype (11, 22), mouse mammary tumor virus (MMTV)[1] expression (35), and MMTV proviral copies (32). It is now accepted that the origin of mammary tumors is monoclonal (10), but how such diversity arises is still unknown. The mechanism could involve either genotypic or phenotypic change. Evidence for a genotypic mechanism, involving mutation followed by cell selection, has been implied from karyotype studies (11, 22, 25) and from changes in exogenous integrated MMTV (32). A phenotypic mechanism would involve stable alteration in the program of gene expression and could result from either selection of phenotypically altered cells (e.g., hormone-unresponsive cells growing selectively under low hormone conditions) or universal change throughout the whole cell population. Little attention has been paid to this latter possibility.

An excellent model system for studies on the progression of tumor cells to steroid autonomy is the androgen-dependent Shionogi 115 mouse mammary carcinoma. This tumor shows androgen-dependent growth properties, and after androgen removal, manifests temporary regression followed by growth of androgen-independent tumors (31). Use of cloned cells

[1] *Abbreviations used in this paper:* +A and −A, androgen responsive and unresponsive, respectively; DC, dextran-charcoal; DME, Dulbecco's Modified Eagle's medium; FCS, fetal calf serum; LTR, long terminal repeat; MMTV, mouse mammary tumor virus; +T and −T, in the presence and absence of testosterone, respectively.

THE JOURNAL OF CELL BIOLOGY · VOLUME 99 OCTOBER 1984 1410–1415

from this tumor, the S115 cell line, provides an in vitro model to study the phenomenon in more detail. Cloned androgen-responsive S115 cells exhibit a positive proliferative response to androgens when maintained as stocks in the presence of testosterone (+A cells) (37). Removal of testosterone (−T) results within 1–3 d in a 50% reduction in growth rate (27, 28) and a total change of all cells from fibroblastic to epithelial morphology (44). The cells develop an increased dependence on anchorage to the substrate for growth (39, 42), an increased density regulation (43), lost ability to grow in suspension culture (44), increased sensitivity to various growth stimuli (43), and an alteration in cytoskeletal structure (5). However, after several weeks of culture in the absence of testosterone, the cells became unresponsive to androgens (−A cells) in terms of cell growth rate, cell morphology, and growth in suspension (39, 43, 44).

Since these dramatic hormone-regulated alterations in cell behavior parallel those changes seen in transformation, we have suggested that some transforming gene could be involved, for example, associated with MMTV (6, 42). MMTV is present in the DNA of S115 cells and no gross genotypic change in proviral copies accompanies the transition from hormone-responsive (+A) to unresponsive (−A) state (6). However, RNA from MMTV is present only in +A and not −A cells, and the loss of MMTV RNA is accompanied by increased methylation in proviral copies in the DNA (6). Thus, in these cloned cells, phenotypic changes occur in the transition to steroid autonomy which are not associated with gross genotypic alteration. In this report, we describe detailed time courses of the phenotypic changes during transition from androgen-responsive (+A) to androgen-unresponsive (−A) state in terms of loss of proliferative response, loss of cell morphological response, loss of MMTV-RNA, and increased methylation of certain MMTV sequences in the DNA. We report attempts to recover hormone sensitivity in insensitive cells.

MATERIALS AND METHODS

Nomenclature of S115 Cells: Stock S115 cells are a cloned cell line maintained in androgen that exhibit a positive proliferative response to androgen. These cells are called +A cells. Long-term maintenance of such cells in the absence of androgen results in the cells becoming unresponsive to androgens, and such cells are then called −A cells. Experimental growth of S115 cells in the presence or absence of testosterone is indicated as +T or −T, respectively.

Culture of Stock S115 Cells: Stock +A S115 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DME) supplemented with 2% dextran-charcoal-treated fetal calf serum (DC-FCS) (FCS from Gibco-Biocult, Glasgow, Scotland), 40 mM HEPES buffer (Sigma Chemical Co., Poole, England), and 3.5×10^{-8} testosterone. DC-treated serum was used in all cultures and this treatment has been described previously (7). Testosterone (Steraloids Ltd., Croydon, England) was dissolved in ethanol and added such that the final concentration was <0.01% ethanol in culture medium. Cells were seeded at 0.1×10^5 cells/ml in 45-ml aliquots in 15-cm plastic tissue culture dishes (Nunc, Denmark) and placed in a humidified atmosphere of 10% carbon dioxide in air at 37°C.

Cells were subcultured at weekly intervals. Cells were suspended by treatment in 5 ml of 0.06% trypsin/0.02% EDTA (pH 7.3) and added to 5 ml of culture medium. The cells were pelleted by centrifugation, resuspended in culture medium alone, counted on a haemocytometer, and replated as above.

Cloned S115 Cells: A single clone was used for all these experiments. The clone of S115 +A cells was selected as fulfilling the criteria: (*a*) it originated from a single cell, (*b*) it was morphologically similar to the original population, (*c*) it exhibited a positive proliferative response to androgen, and (*d*) it possessed androgen receptors (3).

Cells were subcultured until enough were available for experimental use and were then stored frozen in liquid nitrogen in aliquots of 3.5×10^6 cells in 1 ml of DME/10% FCS/10% dimethylsulphoxide (Fisons Scientific Apparatus Ltd., Loughborough, Leicestershire, England).

Stock Cultures for Loss and Recovery of Response Experiments: At the start of each experiment, a new vial of cells was thawed, which ensured that cells were always of the same clone and passage generation. Freshly thawed cells were grown for 2 wk as stock +A cultures and then some cells were switched to medium lacking testosterone. For recovery of response, testosterone was added back to the medium of some stocks. Culture conditions and medium were kept the same throughout except for the presence or absence of testosterone. Cells were assayed from these stock cultures for androgen-responsive growth at regular intervals.

Cell Growth Experiments: Cells were suspended from stock plates by treatment with 5 ml 0.06% trypsin/0.02% EDTA (pH 7.3), added to 5 ml DME/2% DC-FCS/40 mM HEPES buffer, and counted on a haemocytometer. Cells were added to the overall required volume of medium DME/2% DC-FCS/40 mM HEPES buffer at a concentration of 0.1×10^5 cells/ml and plated in monolayer in 5-ml aliquots into 5-cm plastic tissue culture dishes for 24 h. The medium was then changed so that some dishes contained testosterone and some did not. Culture medium was changed routinely every 3–4 d.

Cell Counting: Cells were counted after 24 h to give the plating density and subsequently at least four times in the presence and absence of testosterone, usually after 2, 3, 4, 7, and 9 d. All cell counts were done on triplicate dishes and results were calculated as the mean ± SE.

Cells in monolayer were washed with saline in situ and lysed in 2 ml 0.01 M HEPES buffer/1.5 mM $MgCl_2$ plus four drops of Zaponin (Coulter Electronics Ltd., Harpenden, England) for 5 min. The nuclei released were counted in Isoton (Coulter Electronics Ltd.) in triplicate on a model ZB1 Coulter Counter.

Doubling time of the cells was calculated from at least three early time points of a growth curve; the plot of $\log_{10}$ (cell number) against time was linear and of the form $y = mx + c$. Doubling time was calculated as a function of the slope, m ($\log_{10}2/m$). The error on the slope, S_b, was calculated by the standard statistical formula

$$S_b = \sqrt{\frac{\Sigma(y - y_c)^2}{\Sigma(x - \bar{x})^2} \cdot \frac{1}{n - 2}}$$

where y = actual y value, y_c = estimated y value, x = actual x value, $\bar{x}$ = mean x value, and n = number of values for x and y.

Preparation of Cytoplasmic RNA: A minimum of 3 × 15 cm dishes of stock cells were used for each RNA preparation. Cells were washed in situ with PBS and harvested with a rubber policeman into ice-cold PBS. All procedures were at 4°C from here on. The cells were spun down and resuspended in 0.1 M NaCl/0.001 M EDTA/0.01 M Tris HCl, pH 7.5/0.5% Nonidet P-40 (BDH Chemicals Ltd., Poole, England). The nuclei were then spun out, the supernatant was made 1% in SDS (SERVA, Feinbiochemica GmbH & Co., Heidelberg, Federal Republic of Germany) and extracted with phenol-chloroform (phenol from Fisons was redistilled before use), and the RNA was precipitated in ethanol.

Analysis of RNA by Northern Blotting: RNA was subjected to electrophoresis in 1.5% agarose-formaldehyde gels (30) at 50 μg total RNA per track. The RNA was transferred to nitrocellulose (Sartorius GmbH, Göttingen, Federal Republic of Germany) by blotting in 20 × standard saline-citrate buffer (SSC) and hybridized to 5×10^6 cpm. of ^{32}P-labeled probe per 10 ml. Hybridization was in 1 × SSC, 10 × Denhardt's 0.1% SDS, 50 μg/ml single-stranded calf thymus DNA, and 10% dextran sulphate at 65°C for 16 h. The DNA probe was the cloned 1.4 kilobase pair Pst I fragment of MMTV containing the LTR region. The fragment was cloned into the plasmid pAT 153 (a derivative of pBR 322 [30]) (Biolabs, Beverly, MA) for preparation of large quantities and cut out and isolated from the plasmid before use as a probe. It was labeled with ^{32}P by nick translation (30). Blots were washed at a stringency of 0.2 × SSC, 0.1% SDS for 1 h at 65°C and autoradiographed.

Preparation of DNA: A minimum of 2 × 15 cm dishes of stock cells were used for each DNA preparation. Cells were washed in situ with PBS, harvested with a rubber policeman into PBS, spun down, and resuspended in 10 mM Tris/10 mM EDTA/100 μg/ml proteinase K (BDH Chemicals Ltd.) 0.5% SDS at 37°C for at least 4 h. The viscous solution was extracted with phenol-chloroform and the DNA was precipitated in ethanol. The DNA was dissolved in 10 mM Tris/10 mM EDTA/RNAase A 20 μg/ml (Millipore Corp., Bedford, MA) at 37°C for 2 h. The solution was then made 0.2 M NaCl/0.1% SDS/100 μg/ml proteinase K and incubated another 2 h. The DNA was purified by phenol-chloroform extraction, precipitated in ethanol, and redissolved in sterile 1 mM Tris/1 mM EDTA pH 8 (33, 41).

Methylation Patterns of DNA: High molecular weight DNA was digested with the restriction enzymes Hpa II or Msp I (Biolabs) and the

fragments were separated on 0.8% agarose gels (5 μg DNA per track) and transferred to nitrocellulose as described in detail elsewhere (30). Blots were hybridized to ^{32}P-labeled MMTV-LTR and washed as for the RNA analysis.

RESULTS

Cell Biology

For analysis of loss of responsiveness, S115 cells were kept long-term as stock cultures in the absence of testosterone. To study recovery of responsiveness, we grew some stock cells with testosterone added back to the medium. These cells were then assayed for androgen sensitivity at various time intervals by removing an aliquot of cells from stock cultures and carrying out growth curves over 9 d in the presence and absence of testosterone. Analyses of these growth curves up to and including confluence have enabled us to study the time course of loss of androgen responsiveness in the S115 cells in terms of both proliferative response and saturation density.

Proliferative response has been calculated with respect to the doubling time of the cells in the short-term presence and absence of testosterone (Fig. 1). Stock androgen-responsive +A cells grew much faster in the presence than in the absence of testosterone (week 0, Fig. 1). After 2 wk of androgen withdrawal in stock cultures, the rate of cell growth dropped, especially in the short-term presence of testosterone. However, after 3 wk of androgen withdrawal, the rate of growth of stock cells increased in the short-term absence of testosterone, such that after 4 wk of androgen deprivation the stock cells grew faster in the short-term absence than in the presence of testosterone. Thereafter, the rate of cell growth recovered in both the presence and absence of testosterone until after 9 wk of androgen deprivation, when the stock cells had lost all proliferative response to androgen.

Androgen responsiveness was also studied in terms of saturation density of the cells. We followed changes in the saturation density response over 34 wk of androgen deprivation in stock cultures both by plotting cell numbers per dish at confluence (Fig. 2) and by examining cell morphology under the microscope. Observed cell morphology changes were reflected in the cell number per dish, and can be broadly divided into four stages. (*a*) Stock androgen-responsive +A cells grew to much higher saturation densities in the presence than in the absence of testosterone (week 0, Fig. 2). In the

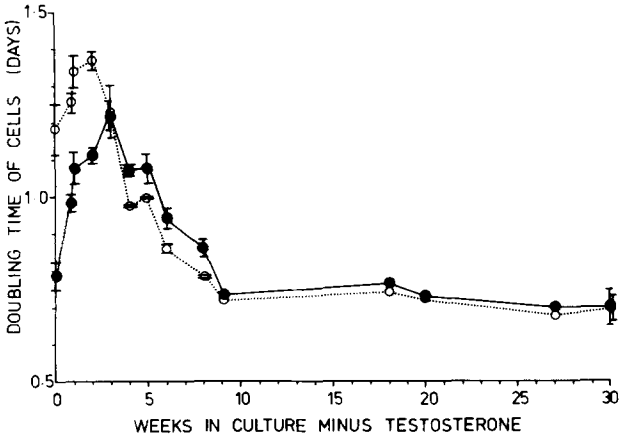

FIGURE 1 Changes in the rate of cell proliferation in monolayer culture in the short-term presence (●——●) or absence (O----O) of testosterone, following increasing periods of long-term androgen withdrawal from stock cultures. Bars represent the error on the doubling times (for calculation, see Materials and Methods).

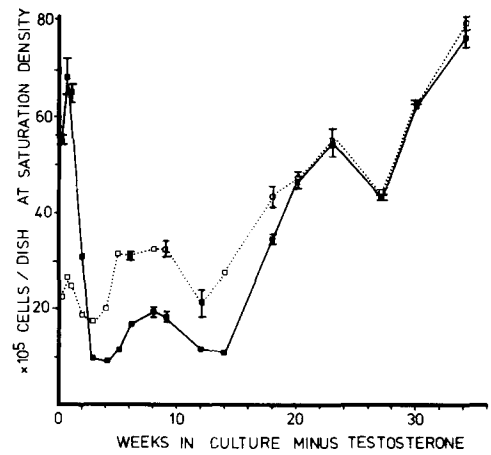

FIGURE 2 Changes in the saturation density in monolayer culture in the short-term presence (■——■) or absence (□- - -□) of testosterone, following increasing periods of long-term androgen withdrawal from stock cultures. Each point represents the mean count of triplicate plates. Bars represent the standard error but no error bars are shown if variation was too low for visual display.

presence of testosterone, the cells at confluence were of rounded fibroblastic morphology (for photograph, see reference 44), forming an uneven spread on the dish, with dense piles of cells in some areas of the dish and empty patches in between. Removal of testosterone resulted in a change to very flattened epithelial morphology (for photograph, see reference 44) and increased anchorage dependence which prevents the cells piling up on top of each other, and this is reflected in the much lower cell number at saturation density. (*b*) After 2 wk of androgen deprivation in stock cultures, the saturation density in the presence of testosterone dropped (Fig. 2). At this stage, the cells lost their ability to revert to the fibroblastic morphology after 1 wk in the presence of testosterone. (*c*) After 3–14 wk of androgen withdrawal in stock cultures, the cells now grew to lower saturation density in the presence of testosterone than in its absence (Fig. 2). The stock androgen-depleted cells maintained an epithelial morphology throughout but changed from a circular to a more elongated form. The elongated cells packed together more closely into an even monolayer than the circular cells and grew to higher saturation densities. After 1 wk in the presence of testosterone, the cells were of the circular epithelial form and grew to lower saturation density than in the absence of testosterone where all cells were of the elongated form. (*d*) After even longer periods of androgen withdrawal in stock cultures, saturation density of the cells both in the presence and absence of testosterone increased, such that after 20 wk of androgen deprivation, the cells had lost all androgen responsiveness in terms of saturation density (Fig. 2). No further morphological changes were observed. The cells grew in elongated form in well-packed even monolayer under all androgen conditions.

These four stages in the loss of androgen responsiveness can be seen clearly in Fig. 3, as the ratio of saturation densities in the presence and absence of testosterone. Attempts to reverse these events are also shown in Fig. 3. Cells grown for 6 wk in the absence of testosterone followed by 6 wk in testosterone-supplemented medium reattained full androgen responsiveness and grew as original +A cells. The longer the cells were maintained without androgen, the longer it took to reverse the process and it may be that once the cells become

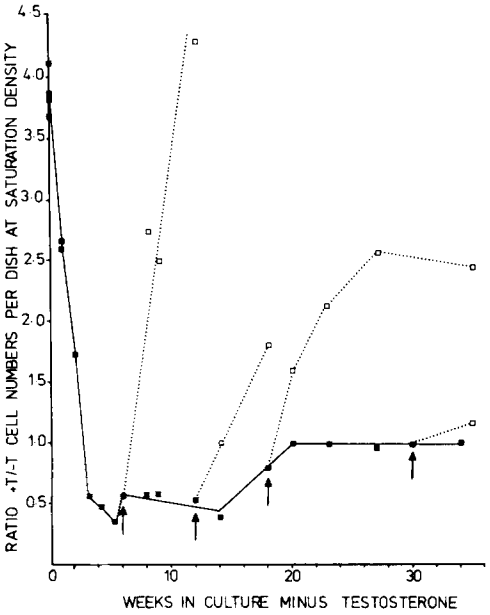

FIGURE 3 Loss and recovery of saturation density response in monolayer culture after long-term withdrawal and readdition of androgen in stock cultures. Results are expressed as ratios of cell numbers per dish in the short-term presence and absence of testosterone, following either androgen withdrawal (■——■) from stock cultures, or androgen withdrawal and then readdition of testosterone (□- - -□) in stock cultures. Arrows indicate the times at which testosterone was readded to stock cultures.

fully unresponsive (−A state) at greater than 20 wk, the process is irreversible by testosterone alone. Fully unresponsive −A cells were kept in androgen-containing medium for 42 wk and reattained no responsiveness at all (data not shown).

Reproducibility of the time course of these events of loss of response has been demonstrated in two separate experiments and also in previous [^{3}H]thymidine labeling studies (3).

Molecular Events

MMTV RNA: The types of MMTV-LTR-related RNA produced in androgen-maintained +A S115 cells have been described previously (6) and are regulated within hours by testosterone (Darbre, manuscript in preparation). A time course of loss of these RNAs during long-term androgen withdrawal revealed total loss after 9 wk (Fig. 4, lanes *1–8*). MMTV-LTR-related RNA was recovered by readdition of testosterone for 1 wk in cells that had been deprived of testosterone for 6 wk (Fig. 4, lanes *4, 9,* and *10*), 12 wk (Fig. 4, lanes *11–14*), or 18 wk (Fig. 4, lanes *15–19*). Recovery was mainly of the 16S RNA, but this may be a detection problem in that the 16S RNA is the major species present. After 34 wk of androgen deprivation, no MMTV-LTR-related RNA was recovered in the cells even after 8 wk of testosterone readdition (Fig. 4, tracks *20–23*). In a separate experiment, fully androgen unresponsive −A cells were kept in androgen-supplemented medium for 42 wk, and although no recovery of androgen-responsive cell biology was attained (see earlier), no MMTV-LTR-RNA was recovered either (data not shown).

Dexamethasone also regulates these MMTV-LTR-related RNAs in the short-term in S115 cells (reference 6 and Darbre, manuscript in preparation), and this response is also lost after long-term androgen deprivation. Cells grown without testosterone for 20 wk were tested after 24 h with (*a*) readdition of testosterone and (*b*) readdition of dexamethasone (10^{-7} M). In neither case was any MMTV-LTR-related RNA recovered (data not shown).

DNA METHYLATION: Methylation patterns of MMTV-LTR-related sequences in the DNA of the cloned +A S115 cells were characterized during a period of androgen withdrawal (Fig. 5). This was done using the isoschizomeric restriction enzymes Hpa II and Msp I, which cut unmethylated DNA at the same sites but differ in their sensitivity to cytosine methylation (40). Since androgen-maintained (+A+T) cells show different patterns with Hpa II and Msp I (Fig. 5), the LTR sequences of S115 +A cells are methylated to some extent. Comparison was then made to Hpa II/Msp I patterns of S115 DNA after increasing periods of androgen withdrawal (Fig. 5). No differences from +A+T DNA were observed until 13 wk of androgen deprivation, when small increases began in methylation of Hpa II-sensitive sites. By 28 wk of androgen withdrawal, two of the small Hpa II restriction fragments were totally missing and after 42 wk all Hpa II fragments below 4 kilobase pairs, except one faint band, were lost.

Reproducibility of these changes in methylation patterns of S115 +A DNA following androgen withdrawal has been established in two separate long-term experiments.

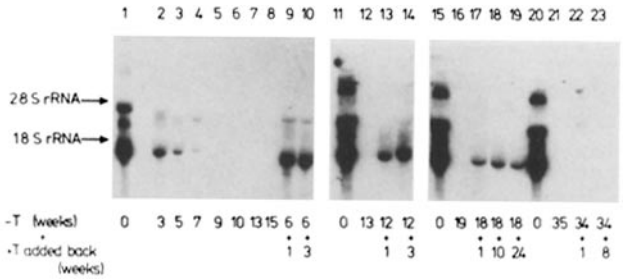

FIGURE 4 Analysis of MMTV-LTR-related RNA in cloned +A S115 cells after various periods of testosterone withdrawal (−*T*) and readdition (+*T*) as indicated. Total cytoplasmic RNA was loaded at 50 μg per lane and autoradiography was for 96 h. Photographic reduction is 91.6%.

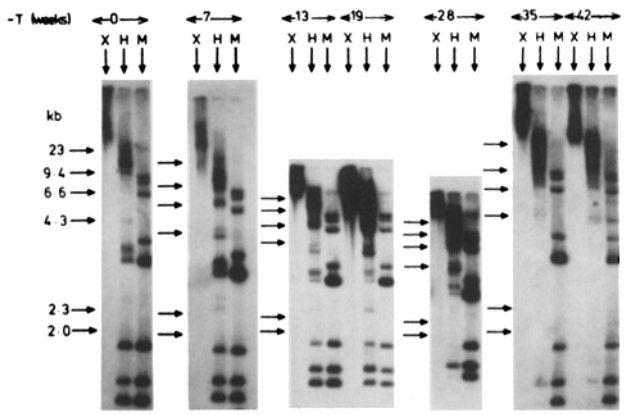

FIGURE 5 Restriction endonuclease analysis of MMTV-LTR-related sequences in the DNA of cloned +A S115 cells following increasing periods of androgen withdrawal (−*T*). DNA was either undigested (*X*) or digested with Hpa II (*H*) or Msp I (*M*) and blots were probed with ^{32}P-labeled MMTV-LTR. Molecular weight standards were provided by a Hind III digest of bacteriophage λ DNA and their positions are indicated by horizontal arrows. Photographic reduction is 91.7%.

The methylation state of MMTV-LTR sequences in fully unresponsive −A cells put back on androgens for 20 wk was also assessed. These studies were carried out in parallel with attempts to recover androgen-sensitive MMTV-RNA and cell biological parameters. Readdition of testosterone did not result in any decrease in methylation (data not shown).

DISCUSSION

We have described here detailed time courses of some cellular and molecular changes during the loss of steroid hormone sensitivity of cells. Growth of cloned androgen-responsive (+A) S115 cells in the prolonged absence of androgen resulted reproducibly in the loss of several hormone-sensitive parameters. Proliferative response was lost after 9 wk of androgen withdrawal, but loss of saturation density response, cell morphology response, and MMTV-LTR-related RNA all took at least 20 wk of androgen deprivation. The data demonstrate that in a cloned hormone-responsive cell line, the transition to steroid autonomy can involve an ordered series of phenotypic changes which are reversible for a certain length of time. These changes cannot be explained by loss of receptor because S115 cells possess androgen receptors in both +A and −A states (3, 28).

Tumors, although monoclonal in origin, acquire marked heterogeneity in cell type (see the beginning of this article). Any studies that investigate the origin of tumor heterogeneity are difficult to perform because response to any stimulus in vivo may result from changes in the relative proportions of subpopulations of cells, and the same argument can be used of an established cell line in which all cells are not identical. Indeed, both the Shionogi tumor (31) and the initial S115 cell line (3) were composed of subpopulations of androgen-responsive and -unresponsive cells. Use of cloned androgen-responsive S115 cells in these experiments has shown that a population of unresponsive cells can be reproducibly obtained from a clone of responsive cells when deprived of hormone in the long-term. The reproducibility of these experiments with cloned cells suggests that selection of genotypically changed (mutated) cells is unlikely to be involved. Alterations along the time course of loss of hormone-sensitive parameters are ordered and begin within 2 wk. It would be unlikely for identical mutations to occur always within such a short length of time. Furthermore, at least one of these phenotypic changes (loss of MMTV-RNA) is not accompanied by gross genetic alterations (6).

This suggests, then, that progression to steroid autonomy may be phenotypic, but the involvement of cell selection in the process will need further study. Cell selection is unlikely to be involved in the cell morphology changes because they are so ordered and uniform. Subpopulations of fibroblastic, circular epithelial, and elongated epithelial cells are not seen under the microscope. Monoclonal cell selection cannot explain the recovery of MMTV-LTR-RNA. MMTV-LTR-related RNA can be recovered after 1 wk of readdition of testosterone. During this 1 wk recovery period, the cells grow more slowly and to lower saturation density in the presence than in the absence of testosterone. It would not be possible for cells that grow more slowly to outgrow a population of cells within 1 wk. However, the general heterogeneity of the response will require further experiments.

After 20 wk of androgen deprivation, the cells became androgen insensitive (−A state) in terms of cell proliferation, cell morphology, and MMTV-LTR-RNA production. At this stage, reversal to an androgen-sensitive state has not been achieved with respect to any of these three parameters. It is interesting to note that the time at which the cells lose response to androgen corresponds to the time at which methylation increases in LTR sequences of the DNA. Since no total methylation of Hpa II-sensitive sites was detected until 28 wk of androgen deprivation, DNA methylation cannot be involved in initial loss of the androgen-sensitive MMTV-RNA production. However, methylation could be involved in final irreversible steps towards androgen insensitivity. But whether it is a direct ultimate control mechanism or merely a consequence of long-term lack of expression remains unclear. DNA methylation has been implicated in eucaryotic gene expression (1, 13, 17, 34), in control of MMTV expression (4), in tumorigenesis in mice (2, 14, 15), in human cancer (12, 16, 20), and in tumor progression to steroid insensitivity (6, 21). Our data presented here lend further support to this latter hypothesis.

These data suggest, then, that progression to hormone insensitivity involves an ordered series of phenotypic changes, which can be reversed up to the time at which the cells lose all cell biological response to androgen and increases in DNA methylation of MMTV-LTR sequences are detected. The extent of the DNA methylation in the total genome of −A cells will now be a subject for further study.

Received for publication 26 March 1984, and in revised form 18 June 1984.

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