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Observations of De Novo Clones of Cytogenetically Aberrant Cells in Primary Fibroblast Cell Strains from Phenotypically Normal Women

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INTRODUCTION

Although it is well known that most fibroblast cell strains derived from primary tissue have a finite life span and that changes in ploidy may occur as these cell strains approach senescence [1], there have been few studies designed to determine how soon after culture initiation stem lines of chromosomally aberrant cells may be observed in fibroblasts from normal individuals. The evolution of abnormal clones has considerable practical importance since the cytogenetically aberrant stem lines derived in culture do not represent the true karyotype of the individual being studied. Fibroblast cultures are routinely used in clinical studies for the evaluation of tissue mosaicism in persons with phenotypic abnormalities and in prenatal diagnosis (via amniocentesis) of high-risk fetuses. Although the time in culture may vary considerably, it is frequently necessary to maintain fibroblast cultures for periods of several weeks before a sufficient number of cells are accumulated for a clinical cytogenetic analysis. During the period of in vitro culture, it is possible that de novo clones of aberrant cells might evolve.

In a recent study of chromosome breakage frequencies in lymphocyte and fibroblast cultures, we observed seven different abnormal stem lines in 36 primary fibroblast cell strains derived from skin of five control women and five women taking oral contraceptives. Five of the aberrant clones were noted in cultures that had been maintained in vitro for periods of less than 70 days. We considered these findings significant because these cultures were all derived from skin biopsies from normal, healthy women. Had these aberrant clones been observed in preparations from persons suspected of having cytogenetic abnormalities, it would have been impossible to know whether these subpopulations of aberrant cells were karyotypically representative of the tissue donor or whether the stem lines arose de novo in culture. A detailed description of the stem lines observed in these cul-

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tures is presented here. A description of the chromosome breakage data is in preparation.

MATERIALS AND METHODS

At intervals of approximately 3–6 months, we obtained a minimum of four sets of blood samples and skin biopsies from each of five control women and from five women who were taking oral contraceptives. The participants, volunteers from local offices, were between the ages of 20 and 40 and had no known phenotypic abnormalities nor family history of congenital malformations. None of the women were taking prescription medications other than the synthetic hormones in the oral contraceptives, and on the days that skin samples were obtained, no illnesses other than colds were reported. (Two biopsies were obtained from women who had colds.) Primary fibroblast cultures were initiated as previously described from 2-mm punch biopsies from each woman [2]. The cell strains derived from these biopsies were maintained in RPMI 1640 culture medium supplemented with fetal calf serum (20%) and antibiotics (50 U penicillin G, 50 μg streptomycin/cm^3). Since our initial purpose was to determine whether serum from women taking oral contraceptives caused chromosome damage, when sufficient cells had been accumulated for cytogenetic studies, replicate flasks from each primary cell strain were exposed to medium containing either fetal calf serum, autologous serum, homologous serum, or serum from a woman taking oral contraceptives (final concentration of serum, 20%). Approximately 24 hr later the cultures were harvested for cytogenetic evaluation.

We attempted to score 100 metaphases from each replicate subculture for induced chromosomal aberrations including chromatid and chromosome gaps, breaks, and all classes of structural inter- or intrachanges. Because the presence of subpopulations of chromosomally aberrant cells might have spuriously raised our breakage estimates, we carefully evaluated slides from each fibroblast preparation for clones of cells bearing morphologically identical lesions. All metaphases with questionable structural rearrangements were photographed and karyotyped for verification. If a specific chromosomal abnormality was noted in three or more metaphases from a replicate series of cultures derived from a single biopsy, we considered these cells to be members of a stem line.

RESULTS

We were able to successfully culture fibroblasts from 19 biopsies from the five control women and 17 biopsies from the five oral contraceptive users. From the various replicate subcultures exposed to either fetal calf serum, autologous serum, homologous serum, or serum from oral contraceptive users, we analyzed 10,202 metaphases for aberrations of chromosome number or morphology. Statistical analyses of these data showed no significant differences in breakage frequencies within replicate cultures exposed to different types of serum, nor among fibroblasts from the control women compared to the oral contraceptive users, suggesting that exposure to synthetic hormones did not affect chromosome breakages in fibroblast cultures.

In the process of scoring these 36 sets of cultures for induced lesions, we observed seven different clones of aberrant cells: five in cultures from the control women and two in preparations from the oral contraceptive users. Since our primary purpose was to evaluate induced aberrations in this large series of cultures, it was not feasible to identify by banding procedures the specific chromosomes involved in the various aberrant stem lines; thus the lesions were classified on the

basis of morphological criteria only. A description of the stem lines in relation to age of culture is presented in table 1, and representative karyotypes from six

TABLE 1

CHROMOSOMALLY ABERRANT STEM LINES OBSERVED IN PRIMARY FIBROBLAST CULTURES FROM CONTROL WOMEN AND ORAL CONTRACEPTIVE USERS

Group and Clone No.	Fibroblast Series*	Days in Culture	No. Cells Scored	No. (%) Cells with Stem Line Rearrangement	Karyotypic Description of Stem Lines
Control women:					
1a	160–163	75	52	38(73)	Modal chromosome number 45 with missing D chromosome
1b	400–403	124	150	~150†(100)	Modal chromosome number 46 with missing no. 3 chromosome and extra "C"-sized chromosome
2	220–223	52	219	6(2.7)	Modal chromosome number 46 with missing D and extra "C" chromosome
3	304–307	69	400	90(22.5)	Modal chromosome number 46 with apparent translocation between long arms of D and short arms of C chromosome
4	176–179	144	90	3(3.3)	Modal chromosome number 46 with dicentric involving no. 3 and C chromosome
Oral contraceptive users:					
5	125–128	41	269	6(2.2)	Modal chromosome number 46 with apparent pericentric inversion of C chromosome
6	348–351	69	400	3(0.8)	Modal chromosome number 46 with dicentric involving two C chromosomes

* Pooled data derived from four replicate subcultures from the same primary fibroblast cell line exposed to either autologous serum, fetal calf serum, homologous serum or oral contraceptive serum during the 24-hr period preceding harvest. No differences were observed in the percentages of the various stem lines in cultures exposed to different sera.

† In a few metaphases the chromosomes were too contracted to positively identify the no. 3 chromosome, making it impossible to determine if the cell had the stem line rearrangement.

of the seven clones are shown in figure 1. As is evident from the table, we observed karyotypically aberrant stem lines in cultures ranging in age from 41 to 144 days. These stem lines comprised varying percentages of the cell populations, ranging from less than 1% to virtually 100%. A brief description of the various clones follows.

Clone 1a. We successfully cultured four separate biopsies from one control woman; from fibroblasts from one biopsy (evaluated after 75 days in culture) we observed a major stem line of cells that was hypodiploid with a missing D chromosome. The overall morphology of this culture was quite poor, and we were able to score only 52 metaphases from the four replicate subcultures. The 45,XX,—D karyotype was the predominant karyotype in all four cultures.

Clone 1b. From a second biopsy obtained from the same woman, analyzed after

FIG. 1.—Representative karyotypes of six aberrant clones (1a, 1b, 2, 3, 4, and 5) observed in primary fibroblast cell lines from five phenotypically normal women. See table 1 for description of stem lines.

124 days in culture, we observed a second major clone of cells having a completely different karyotype. In all four replicate subcultures all metaphases that could be microscopically analyzed had only one no. 3 chromosome and an "extra" chromosome the size of a C. In some metaphases an atypical unmatched "C"

chromosome with unusually short short arms could be identified (see fig. 1), suggesting that the apparent pseudodiploidy in these metaphases may have resulted from a deletion of the short arms of a no. 3 chromosome, giving rise to a chromosome resembling a C. It is also possible that the stem line may have truly been pseudodiploid, that is, monosomic for a no. 3 and trisomic for a C.

Clone 2. In a preparation cultured for 52 days from a second control woman we identified six metaphases out of 219 cells that had a 46 count, with five D chromosomes and an extra chromosome in the C group. This stem line may have been pseudodiploid, or the lesion could have resulted from a structural rearrangement—that is, pericentric inversion of the D chromosome or a reciprocal translocation between a C chromosome and the short arms of a D.

Clone 3. A complex lesion involving a reciprocal translocation between the long arms of a D chromosome and the short arms of a large C chromosome was observed in 90 of 400 metaphases from a 69-day-old culture from a third control woman. In metaphases bearing this rearrangement, a small acrocentric chromosome approximately half as large as the G group chromosomes was consistently present in conjunction with only five identifiable group D chromosomes. In metaphases with good morphology, we could identify a large metacentric chromosome approximately the size of chromosome 3. Presumably the extra "3" was derived from a reciprocal translocation of the long arms of the D to the short arms of the C.

Clone 5. We noted one other symmetrical aberration in a 41-day-old culture from a woman taking oral contraceptives. In this preparation six of 269 metaphases had a chromosome which on first impression appeared to be a B with a deletion of approximately one-half of the short arms. However, in metaphases with extended chromosomes in which the group B chromosomes were distinct, we could identify both pairs of chromosomes 4 and 5, but only 15 normal-appearing C chromosomes. We thus concluded that the "marker" chromosome, with long arms comparable in size to the B chromosomes, was derived from a pericentric inversion of a large C chromosome.

Clones 4 and 6. We observed minor stem lines bearing asymmetrical interchanges in one culture from a control woman and in one preparation from an oral contraceptive user. Three dicentric chromosomes were identified in each preparation. In cultures from the control woman, two C group chromosomes were involved in the formation of the dicentrics, whereas in the oral contraceptive user the dicentrics were derived from a no. 3 and a C chromosome. A hypodiploid metaphase showing the second dicentric is shown in figure 1. Although we cannot be absolutely sure that the three cells bearing the dicentrics in each of these cultures were members of clones of cells bearing these lesions, this interpretation is probably accurate, since it would be very unlikely to randomly observe in a single preparation three dicentrics derived from what appeared to be the same two chromosomes.

DISCUSSION

Since the original purpose of our study was to determine whether synthetic hormones caused chromosomal damage in fibroblasts, it was necessary that we select for cytogenetic evaluation a group of clinically normal women whose family history gave no indication of possible inherited chromosomal abnormalities. To give validity to our chromosome breakage estimates, we attempted to score for cytogenetic lesions 100 metaphases from each of four replicate subcultures from each primary cell strain. This experimental design gave us an excellent opportunity to observe the presence of cytogenetically aberrant clones in fibroblast cell strains from phenotypically normal individuals. We feel that our findings have some relevance for clinical studies employing fibroblast cultures for karyotypic determinations in persons suspected of having chromosomal abnormalities. Obviously the presence of abnormal stem lines in clinical preparations could lead to erroneous interpretations of the in vivo chromosome complement of the individual being studied.

A few points regarding our preparations should be mentioned. That the seven stem lines observed in these 36 primary cultures arose as an artifact of in vitro culture is suggested by several observations. First, we had no reason to suspect aberrant karyotypes in any of the 10 women studied since their clinical and family history was completely negative. Second, lymphocyte cultures from all women were found to be completely normal with no evidence of congenital mosaicism detected in any of several repeated lymphocyte preparations. Third, the clones appeared in fibroblasts derived from single biopsies; fibroblasts from different biopsies obtained from the same women did not have the same stem lines. For example, we obtained four separate biopsies from one woman and observed two totally different major clones in fibroblasts from two of the preparations (clones 1a and 1b) and completely normal karyotypes in fibroblast cultures derived from two other biopsies obtained at different times. The fact that we obtained skin biopsies from 10 women and observed aberrant clones in fibroblast preparations from six of the women suggests that the evolution of stem lines is a fairly common occurrence in this cell type under the culture conditions that we used.

The length of time our cultures were maintained in vitro before cytogenetic evaluations were made varied considerably (from 41 to 144 days). The long culture time in many preparations is explained in part by the fact that the fibroblasts in some cultures would not undergo mitosis within the specified interval after exposure to various test sera. If all four replicate flasks in a single experiment did not have sufficient mitoses for analyses, we later repeated the entire set of serum experiments. Many of our cultures could have been analyzed several weeks earlier had we not attempted to define the effects of various sera on aberration frequencies. Even with the imposition of the serum studies, several of our cultures were harvested within 6–10 weeks after the initial biopsies were obtained. Many clinical fibroblast cultures derived from skin biopsies are maintained in

vitro for comparable periods of time and under certain conditions might be as likely to evolve de novo clones as fibroblasts from these normal women.

The percentage of stem lines in these 36 primary cell strains is considerably higher than that observed by many laboratories involved in clinical evaluations of fibroblast cultures. The reasons for the high frequency of clones in our cultures are not clear. No doubt we detected the four minor stem lines (clones 2, 4, 5, and 6) by virtue of the fact that we analyzed large numbers of metaphases for aberrations; and from a clinical standpoint, it is unlikely that the degree of mosaicism in these four cell strains would be significant. Even disregarding these four series of cultures, three of the remaining 32 preparations (10% of our sample population) had major stem lines with karyotype alterations. It is possible that unidentified factors conducive to the evolution of aberrant clones were operable in our specific culture system. Regarding our culture technique, to our knowledge, the only major deviation from "standard procedure" was our use of RPMI 1640 culture medium (developed for use in propagating leukemic cells) in lieu of more commonly used "fibroblast" culture media (i.e., minimum essential medium, Ham's F-10, F-12). Since we do not have a comparable series of cultures maintained in other media, we have no means of determining whether our results were affected by our choice of media. A second culture condition may have influenced our findings. In a recent biochemical evaluation of mycoplasma contamination in 20 cell cultures derived from amniotic fluid, Schneider et al. [3] observed that 50% of the metaphases in one of 11 cultures shown to be infected with mycoplasma had consistent multiple translocations. Although we did not monitor all of our cell strains for mycoplasma contamination, microbiological testing of several lots of medium, serum, and cultures yielded negative findings. However, since Schneider et al. [3] obtained positive results for mycoplasma in 11 of 20 cultures tested using biochemical methods and negative results in the same cultures tested using microbiological methods, we cannot exclude the possibility that some of our cell strains may have been infected with mycoplasma, resulting in the evolution of aberrant clones.

Regardless of the etiology of the aberrant stem lines in our cultures, our findings give further documentation to the fact that under certain culture conditions high frequencies of de novo clones can evolve in fibroblast cell strains from clinically normal persons; that in some instances the clones may comprise virtually 100% of the recoverable cell population; and that clones of cells resulting from nondisjunction as well as structural rearrangements may arise in culture. These findings should be taken into consideration in interpretation of data derived from clinical fibroblast cultures.

SUMMARY

In a recent study of chromosome breakage frequencies in 36 primary fibroblast cell strains derived from skin from 10 phenotypically normal women, we observed seven different clones of cells having consistent chromosomal abnormalities. Five of the stem lines were noted in cultures from "control" women and two in fibro-

blasts from women taking oral contraceptives. We observed aneuploid clones as well as stem lines bearing structural abnormalities (e.g., translocations, inversions). The various aberrant clones were found in cultures ranging in age from 41 to 144 days and comprised varying percentages of the cell populations ranging from 0.8% to virtually 100%. The possible evolution in culture of clones of cells having aberrant karyotypes should be considered in interpreting findings from fibroblast cultures initiated for clinical evaluations.

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