Production of G and C Banding with Progressive Trypsin Treatment

S. MERRICK, R. S. LEDLEY, AND H. A. LUBS^[12]

Department of Pediatrics, University of Colorado Medical Center, Denver, Colorado, and National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D. C., USA

Extract

Human metaphase cells were treated sequentially with trypsin. Photographs of the cells stained before and during this treatment showed first Giemsa (G) banding then a gradual decrease in prominence of G bands and the appearance of C bands. These results were confirmed using computer-generated density curves.

Speculation

These results are more consistent with loss or change in configuration of chromosomal protein being the primary mechanism by which G and C bands are produced than denaturation-renaturation. Unless trypsin banding is carefully controlled a combination of G and C banding will be produced.

Introduction

The use of chromosomal banding techniques can greatly enhance the value of routine cytogenetic evaluation of children with mental retardation and congenital anomalies. Three laboratories have recently reported the production of striking banding patterns in human chromosomes after treatment with trypsin [2, 8, 10]. Both in their observations and in our own early trials with the technique, the details of the banding were variable and considerable swelling and destruction of the chromosomes were produced. In order to standardize this treatment for our own use, to use it as an investigative tool in determining the mechanism of banding, and to determine whether G banding [9] produced by this and other techniques were identical, a series of controlled experiments were undertaken. The unexpected production of C banding [9] after progressive disappearance of G banding with longer periods of trypsin treatment prompts this brief report.

Cytogenetic Techniques

Lymphocytes were grown for 66-70 hr in Gibco McCoy's 5A medium with 15% fetal calf and phytohemag-

glutinin. A final concentration of colcemide of 0.1 μ g/ml was employed for 2 hr. Hypotonic treatment was carried out for a total of 10 min with 0.075 M KCl at room temperature. After two changes in 3:1 methyl alcohol-acetic acid, the cell suspension was dropped onto a clean glass slide on which there was a layer of cold water. Spreading of metaphases was carried out first by blowing vigorously at the cell suspension at a right angle to the slide and then by placing the slide directly on a hot plate for 1.5-2 min. The hot plate was set to maintain a beaker of water at 60-65°. This method of drying is critical for obtaining banding with the Giemsa 9 technique. The slides were then stained with Giemsa at pH 9.0 as previously described [7] or by the acetic-saline-Giemsa (ASG) technique [1]. The following destaining procedure was used. Xylene (2 min), xylene-absolute alcohol (50:50) (1 min), 100% ethyl alcohol (2 min), 95% ethyl alcohol (2 min), and 70% ethyl alcohol (2 min). Initially, in half the slides 1% concentrated HCl was added to the final 70% alcohol step without affecting the results, but with improved destaining. This was, therefore, subsequently

employed. The slides were air-dried at room temperature.

Two types of trypsin were employed with comparable results. Difco trypsin (Catalog No. 053) was diluted to 2.5% stock solution in sterile distilled water and then further diluted with phosphate-buffered saline solution (PBS) buffer at pH 7.39 for use (0.025% final concentration). Slides were then rinsed with 95% and 70% ethyl alcohol and stained conventionally with Giemsa. Comparable results were obtained with more highly purified trypsin obtained from Worthington Biochemicals but the concentration used was lower (0.001% final concentration). These preparations were assayed for DNAase activity [4] and found to have no activity in the concentrations employed. At more con-

centrated levels (2.5%), however, DNAase activity was found with Difco trypsin; slightly less activity was found with Worthington trypsin.

Dual, triple, or quadruple karyotypes provided the principle means of comparing banding patterns; *i.e.*, the same cell treated in various ways was photographed after each treatment and karyotypes were prepared in which the differently stained versions of the same chromosome were placed in sequence (as in Fig. 1).

Experimental Procedures

Two types of experiments were carried out, "sequential" and "prolonged" treatment. In the simplest se-

Fig. 1. Chromosomes 1, 9, 16, and Y from two cells, showing loss of G and appearance of C banding. From left to right, for each homolog, banding after Giemsa 9 staining, 4, 12, and 24 min of trypsin treatment is shown. In 1 and 16, the secondary constriction (h) regions and adjacent centromeres are stained darkly and the area stained does not change during the progression from G to C banding. In 9 the h region becomes darkly stained and the C band includes both the h region and the darkly stained centromere. In the Y chromosome the distal two-thirds of the long arm becomes darkly stained after 24 min trypsin treatment, but gradual darkening is evident.

quential treatment, slides were initially stained either by the Giemsa 9 or ASG technique, destained as described above, treated with trypsin for 4 min, and restained with Giemsa at pH 6.8. In the second type of sequential treatment, slides were initially stained with Giemsa 9, treated with trypsin for 4 min, stained with conventional Giemsa, destained, treated again with trypsin, restained, and destained as above for varying periods of 6, 8, or 12 min. A total of 30 min of trypsin treatment was employed. In control experiments carried out as above, but without trypsin, no change in banding was observed. In the "prolonged" trypsin treatment, slides were stained with the Giemsa 9, destained, and treated once for varying periods ranging between 10 and 30 min of trypsin treatment, without intervening staining or destaining, in order to determine the time necessary to obtain C banding directly. The average time was 20 min, but varied between 15 and 30 min.

Results

With an average of 2–4 min trypsin treatment patterns were obtained that were identical with both initial Giemsa 9 or ASG treatment (Fig. 1). Giemsa 9 and ASG banding had previously been shown to be identical [6]. This experiment has been repeated many times and it was clearly demonstrated that, with the reservations discussed below, the present 4-min trypsin treatment produces G banding, as defined by the Paris Conference [9]. Only a partial karyotype is shown, but the banding changes were identical for each chromosome. As shown in Figures 1 and 2, progressive trypsin treatment produced first a swelling of the chromosomes, followed by gradual loss of Giemsa banding and the appearance of C banding. The latter effect can be seen most strikingly if chromosomes 1, 9, 16, and the Y chromosome from the same cell are placed side by side after each of the sequential treatments. This effect was most marked in the Y chromosome, which initially stained with a medium intensity without marked Giemsa banding. The gradual appearance of the dark Giemsa staining at the end of the long arm of the Y chromosome is readily apparent and easily repeatable. The second major change appeared in chromosome 9; the G banding pattern in the initial portion of the long arm had a light region in the secondary constriction area (h), next to a dark centromere. This light staining band became dark staining after an average of 30 min trypsin treatment with the result that there was a single larger dark area including both the centromere and h regions, *i.e.*, the C band. It was

necessary, however, to use progressively increasing staining times (from 4 min to 8–10 min) with conventional Giemsa stain as the treatment with trypsin progressed.

These results have now been confirmed with an adaption of the FIDACSYS system of automated computer analysis which generated density curves for G-banded cells [5]. However, after only 3 min trypsin treatment the h region in 9 was more dense (Fig. 3). This introduces an element of uncertainty in trypsin G banding: it is likely that in practice it will contain an element of C banding. This was not noticed by direct observation.

There are also drawbacks to the routine use of trypsin for C banding, since there was sufficient variation from slide to slide and culture to culture in the time necessary to produce optimal C banding, that a reliable standard routine could not be developed. Since sequential observation was the best means of obtaining C banding this approach was more time consuming than other C banding techniques [6] and, therefore, has not been used routinely. Longer treatment resulted in apparent destruction of chromosomes, with only a "ghost-like" shell remaining.

Discussion

These results have important implications both for our understanding of the mechanisms of banding and for the possible routine use of trypsin to produce banding in cytogenetic laboratories. The most frequently given explanation for both G and C banding involves denaturation and differential renaturation of DNA in certain regions. The methods used in these several experiments are not likely to affect strandedness (*i.e.*, denaturation or renaturation) of DNA significantly and whatever changes are produced are most likely due to loss of chromosomal protein. The most direct conclusion that can be reached is that banding is predominantly influenced by the presence or configuration of proteins which are lost or changed during trypsin treatment. In the case of G banding these changes may be minimal, although still sufficient to produce banding. C banding may be best explained by a combination of loss of proteins over the C regions in 9 and Y, permitting these C regions to be stained, in conjunction with more profound changes in the non-C regions, *i.e.*, protein loss and secondary loss of DNA following removal of proteins. This explanation is supported by the fact that these non-C regions either did not stain or required an increasing long staining time after trypsin. More prolonged trypsin treatment ap-

Fig. 2. One of the above cells, showing loss of G banding and appearance of C banding in all chromosomes. Arrows indicate chromosomes 9, Y, and 1 (left to right) in which C banding is particularly prominent.

Fig. 3. Density curves obtained from 35-mm negatives for both 9 chromosomes (same cell) are shown. These demonstrate loss of G and appearance of C bands. Treatment consisted of initial ASG banding followed by 3, 8, 15, 24, and 35 min trypsin treatment. Even after 3 min treatment the h region is more pronounced, as shown by the higher curve in the secondary constriction region.

peared to cause still more profound disruption of the chromosomes. The biochemical studies carried out by Comings [3] provide more direct evidence of loss of DNA and protein during trypsin treatment.

Although G banding patterns can be obtained in a high proportion of cells with trypsin treatment the technique is somewhat awkward to use routinely, since the optimal time of trypsin treatment varies somewhat from culture to culture and slide to slide. This necessitates sequential treatment of the type employed here in order to obtain optimal banding. As reported [8], this approach can be facilitated by direct observation of unstained metaphases by phase microscopy. If bands are not observed, additional trypsin can be employed. (Metaphases prepared for G banding by the Giemsa 9 technique to not show banding under these conditions, and the production of bands observable by phase microscopy probably indicates a degree of chromosome destruction.) Since this procedure is time consuming, we find that direct utilization of either the Giemsa 9 or

ASG technique is simpler and, therefore, preferable. Moreover, if the trypsin treatment is not carefully controlled, loss of Giemsa bands, alteration in the size of Giemsa bands, or varying combinations of Giemsa and C banding will be produced. For many laboratories, however, which are having difficulties with other Giemsa banding techniques, this technique may prove a useful means of obtaining G- and C-banded preparations. Extreme care and careful standardization, however, will be necessary, as well as an awareness of the several pitfalls.

Conclusions

The identity of Giemsa 9, ASG, and trypsin bands following brief trypsin treatment was shown. With longer periods of trypsin treatment, G bands were shown to disappear progressively and C banding to appear. It is concluded from these experiments that progressive loss of chromosomal protein likely occurs

with secondary loss of DNA following longer trypsin treatment. The production of G and C bands, therefore, is primarily brought about by various treatment effects on chromosomal proteins, either loss or change in configuration. Denaturation and differential renaturation of DNA is an unlikely explanation for C and G banding.

References and Notes

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12. Requests for reprints should be addressed to: H. A. LUBS, M.D., University of Colorado Medical Center, Denver, Colo. (USA).
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