DNA methylation prevents transfection of genes for specific surface antigens

(trophoblast/choriocarcinoma/gene regulation/5-azacytidine)

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Contributed by Leonard A. Herzenberg, July 11, 1988

ABSTRACT Sperm and trophoblast are among the few nucleated human cells that do not express HLA class I antigens. DNA methylation, which is proposed to be a tight mechanism of regulation, may be necessary to turn off these genes. We have investigated the transfectability of HLA class I genes and of the genes for the T-cell differentiation antigens Leu-1 (CD5) and Leu-2 (CD8) in mouse L cells by using human sperm cells and choriocarcinoma cell lines, tumors of trophoblastic origin, as sources of DNA. It was found that DNA from one choriocarcinoma line (JAR) does not transfect genes for HLA, Leu-1, or Leu-2 and that DNA from two other choriocarcinoma lines (BeWo and Ima) transfects only some of the surface markers. Sperm DNA transfects genes for all the surface antigens tested except Leu-1. DNA from control cells and from the line SCH transfects all the markers studied. Southern blots show that all cell types contain apparently intact genes encoding HLA, Leu-1, and Leu-2 and reveal differences in the DNA methylation patterns of genes from different sources of DNA. We treated JAR (the cell line with the lowest transfecting ability) with 5-azacytidine and obtained demethylation of its DNA. This demethylated DNA transfects genes for both HLA class I antigens and Leu-2. Further culture of JAR cells in the absence of 5-azacytidine results in remethylation of their DNA and decreased ability to transfect these surface antigens. These findings indicate that DNA methylation affects the efficiency of transfection of surface antigen genes in L cells.

The role of DNA methylation in gene regulation is still unclear (1). DNA methylation is likely to interfere in a dual manner with protein-mediated regulation, since the state of methylation can determine chromatin conformation in transfected DNA (2) and the structure of chromatin can predetermine sites of methylation in sperm DNA (3). Functional studies on the role of DNA methylation in gene regulation would be greatly facilitated in systems where protein-mediated regulation does not severely interfere. Transfection of naked genomic DNA in eukaryotic cells appears to be suitable for such studies, since proteins are removed from the DNA during DNA extraction, but DNA methylation is retained (4). In this paper we have investigated the transfectability of genes for HLA class I antigens and of the T-cell differentiation antigens Leu-1 (CD5) and Leu-2 (CD8) by using DNA isolated from human choriocarcinoma (CC) cell lines and sperm. Sperm and trophoblast (cells of origin of CCs) are apparently among the few nucleated cells in the body that do not express HLA class I antigens (5–7). We hypothesized that the genes encoding the antigens studied might be turned off because they are methylated in control regions. Indeed, we found that transfection of specific surface antigens is prevented by DNA methylation.

MATERIALS AND METHODS

Cell DNA Extraction. High molecular weight DNA was obtained from the human CC lines JAR, Ima, BeWo, and SCH (previously called Enami). DNA was also purified from human sperm cells, from the T-cell lymphoma JM, and from peripheral blood leukocytes. Spermatozoa and leukocytes were obtained from the same donor to avoid restriction fragment length polymorphism differences. DNA was extracted either by conventional methods of sodium dodecyl sulfate (SDS)/proteinase K (Boehringer Mannheim) lysis followed by banding on a cesium chloride gradient (8) or by our procedure of cell lysis by 4 M guanidinium thiocyanate (Kodak) followed by banding over 5.7 M cesium chloride (unpublished observations). Since we obtained higher transfection efficiency with the guanidinium method compared with conventional methods (unpublished observations), most transfections were performed with DNA isolated with guanidinium.

DNA Transfection. The calcium phosphate coprecipitation technique was used (9). Briefly, the day before the transfection 10⁶ thymidine kinase minus L (LTK-™) cells were seeded in each dish. The transfections were performed with 20 μg of genomic DNA coprecipitated with 1 μg of plasmid containing the thymidine kinase gene. Hypoxanthine/aminopterine/thymidine (HAT) medium was used to select cells that expressed the thymidine kinase gene and the co-transfected high molecular weight DNA.

Immunofluorescence. Fluorescence analyses and sorts were made on a modified fluorescence-activated cell sorter (FACS II, Becton Dickinson), used essentially as described (10). To improve the detection of transfected cells stained with fluorescein-coupled antibodies, subtraction of cell autofluorescence was performed as described (11).

Antibodies. Fluorescein isothiocyanate-conjugated anti-Leu-1 (CD5) and anti-Leu-2a (CD8) antibodies were provided by Becton Dickinson. The anti-Trop-1 (12) and W6/32 (anti-HLA class I, recognizing a framework determinant on HLA-A, -B, and -C molecules) (13) antibodies were conjugated with fluorescein isocyanate by using conventional methods (10). Cell staining was performed as described (10).

DNA Blot Analysis. This was performed by the method of Southern (14). Restriction enzymes were purchased from New England Biolabs and from Boehringer Mannheim. The probes used were pHLA-B7 (15), pBR-i (Leu-2 cDNA) (16), and pTI-2.2 (Leu-1 cDNA) (17).

5-Azacytidine (AzaC) Treatment. JAR cells were cultured in medium with 10 μM AzaC for 4 days—i.e., two population-doubling times—and then cultured in the same medium without AzaC. DNA was extracted at various times after the treatment (5–12 days, 6–8 weeks, or 5–6 months) (see Table 2) and used in transfection experiments.

Abbreviations: AzaC, 5-azacytidine; CC, choriocarcinoma.
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RESULTS

Table 1 shows the frequencies of transfection of the surface antigens studied (HLA class I, Leu-1, Leu-2, and Trop-1) with DNA from several CC lines as well as from sperm, leukocytes, and JM (a T-lymphoma cell line).

Interestingly, DNA from JAR cells does not detectably transfect genes for HLA, Leu-1, or Leu-2 antigens. DNA from BeWo and Ima cells transfect only some of the markers. DNA from sperm, which does not express any of the antigens studied as detected by fluorescence-activated cell sorter analysis (data not shown), transfects genes for HLA class I antigens and Leu-2 but not Leu-1. SCH cell, leukocyte, and JM cell DNAs transfect all the markers investigated. All the DNA sources we tested transfected the gene for Trop-1, although this antigen is expressed only on some CC lines. The latter findings are consistent with results we obtained in a murine system: DNA from many mouse tissues that do not express various surface antigens nevertheless transfected L cells with genes for these antigens (9).

There is a correlation between transfestability of HLA class I and of the T-cell differentiation antigens with expression of HLA class I molecules by the CC lines. Indeed JAR DNA has the lowest transfection efficiency for the markers studied and JAR cells do not express any HLA, whereas SCH cell DNA does transfect all the markers and SCH cells express normal levels of HLA. BeWo and Ima cells express intermediate levels of HLA (6) and DNAs from these cells have intermediate transfection abilities.

The lack of transfection of specific surface markers is unlikely to be due to poor transfection efficiencies because (i) DNA from all sources reproducibly gave similar numbers of HAT-resistant transfected colonies and transfected Trop-1 at high frequency and because (ii) transfections were performed with multiple DNA preparations from each cellular source and the various DNAs were tested up to nine times with very consistent results.

Southern blots performed after digesting the DNA with a number of restriction endonucleases reveal that the genes for Leu-1, Leu-2, and HLA are present in all the cell lines tested and do not show detectable restriction pattern changes compared with DNA from blood leukocytes (data not shown). The only exception found so far is represented by a BamHI restriction fragment length polymorphism of one of the two copies of the cell line Leu-2 DNA (data not shown).

By using the enzymes Msp I and Hpa II [isoschizomers differentially sensitive to DNA methylation (18)], different patterns of methylation were found in the various sources of DNA (Fig. 1). Similar data were obtained with the methylation-sensitive enzymes Cfo I and Hha I (data not shown). A number of criteria indicate that the different band patterns observed in Southern blots were due to different states of methylation of the various genes and were not artifacts caused by partial digestions: (i) Agarose gels stained with ethidium bromide after DNA electrophoresis showed complete digestion of all DNA samples used for final Southern blot analysis. (ii) By using fixed amounts of Hpa II or Hha I (10 units per µg of DNA), DNA digestion was already

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Table 1. Transfection efficiency of the various sources of DNA

<table>
<thead>
<tr>
<th>Gene transfected</th>
<th>JAR DNA</th>
<th>BeWo DNA</th>
<th>Ima DNA</th>
<th>SCH DNA</th>
<th>Sperm DNA</th>
<th>Leukocyte DNA</th>
<th>JM DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA</td>
<td>0/16</td>
<td>0/14</td>
<td>5/8</td>
<td>3/4</td>
<td>9/12</td>
<td>6/7</td>
<td>ND</td>
</tr>
<tr>
<td>Leu-1</td>
<td>0/11</td>
<td>0/8</td>
<td>0/8</td>
<td>1/5</td>
<td>0/12</td>
<td>4/9</td>
<td>11/16</td>
</tr>
<tr>
<td>Leu-2</td>
<td>0/16</td>
<td>3/14</td>
<td>0/8</td>
<td>3/6</td>
<td>14/14</td>
<td>15/15</td>
<td>15/18</td>
</tr>
<tr>
<td>Trop-1</td>
<td>25/29</td>
<td>8/8</td>
<td>8/8</td>
<td>6/6</td>
<td>7/9</td>
<td>15/15</td>
<td>9/16</td>
</tr>
</tbody>
</table>

HLA was not expressed in JAR cells or sperm. HLA expression was low in BeWo and Ima cells, was intermediate in SCH cells, and was high in leukocytes and JM cells. Cumulative results of 14 experiments are presented. ND, not done.
FIG. 2. DNA methylation patterns of the Leu-2 gene in JAR cells, untreated and at various times after 4 days culture in medium with AzaC. DNA samples (10 μg) isolated from these cells were cleaved with restriction enzymes, electrophoresed on 0.8% agarose gels, blotted, and hybridized with Leu-2 cDNA probe (16). DNAs were digested with BamH1/Hpa II or BamH1/Msp I as indicated. Lanes: –, untreated JAR cells; 5d, 5 days; 8w, 8 weeks; 12d, 12 days; 6w, 6 weeks; 5m, 5 months after treatment. Results from two independent experiments are included. Lanes 5d–8w are from experiment 1 and lanes 12d–5m are from experiment 2.

iciency of the DNA extracted after growth in AzaC was highest 12 days after the treatment and remained considerable after 6–8 weeks until it finally dropped after 4–6 months (Table 2). The treatment with AzaC was repeated in a second experiment. Transfections of the demethylated DNA at similar times gave similar results. The patterns of demethylation and remethylation of JAR DNA were different in the two experiments (Fig. 2). Thus, both the demethylation caused by AzaC and the remethylation that follows do not maintain a strictly predetermined pattern.

We analyzed JAR cells by surface staining at various times after the treatment with AzaC. No expression of HLA class I and Leu-2 antigens could be detected between 5 days and 6 months after the treatment.

DISCUSSION

In this article we show that differential DNA methylation prevents transfection of some surface antigen genes. This conclusion is based on the following findings. Genes for HLA class I, Leu-1, and Leu-2 antigens cannot be transfected in L cells by using DNA from certain human CC lines, and the gene for Leu-1 cannot be transfected by human sperm DNA. We have obtained similar numbers of HAT-resistant colonies and similar transfection frequencies of the Trop-1 gene with all DNA; thus artifacts of transfection are unlikely. Southern blots demonstrate the presence of apparently intact genes coding for the surface antigens studied in all sources of DNA. These genes are methylated differently in the different cell types used. No clear relationship, however, can be established between patterns of methylation at specific sites and transfection efficiency.

Direct evidence that methylation is responsible for the lack of transfection of genes for HLA class I and Leu-2 was obtained by reacting the transfectability of these genes by culture of JAR cells with AzaC, a DNA demethylating agent. JAR, the cell line possessing the lowest transfection ability, was treated with AzaC and DNA was extracted at various times after the treatment. Demethylated JAR DNA transfected genes for HLA and Leu-2 efficiently and, as remethylation of the DNA continued in culture, the efficiency of transfection of these markers decreased. DNA extracted 12 days after the AzaC treatment has the highest transfection efficiency. Maximum DNA hypomethylation, however, is found at day 5. AzaC, when incorporated in the DNA, induces the appearance of fragile sites that can result in single-strand breaks (21). Thus, the AzaC present in the DNA at day 5 may cause structural damage to the genes transfected. The concomitant low transfection efficiency of Trop-1 is consistent with this explanation. The DNA extracted 4–6 months after the AzaC treatment transfects all the markers studied at low efficiency. DNA hypermethylation is the likely cause of this finding. Preliminary data indicate further reactivation of the HLA and Leu-2 genes after a second demethylating treatment of JAR cells with AzaC, performed 6 months after the first treatment (unpublished observations).

Direct effects of AzaC on DNA, RNA, and protein synthesis (22) did not influence the outcome of transfections performed with DNA obtained from cells treated with AzaC, since there is essentially no carry-over of the drug to the transfected cells.

DNA methylation is the most likely cause of the low transfection ability we observed since it is a covalent modification of the DNA and it is essentially retained after transfection (5). A formal alternative explanation is the presence of structural damage in the genes that are not transfecable. This is highly unlikely in sperm cells, since it would imply nonfunctional Leu-1 genes in the germ line while Leu-1 is functional in leukocytes from the same individual. Moreover, it would be necessary to postulate that back mutations caused by AzaC can reactivate HLA and Leu-2 genes in JAR cells. This possibility is not consistent with the high frequency of transfection of the genes studied and with the cycles of reactivation/inactivation that follow AzaC treatments.

No expression of HLA class I, Leu-1, and Leu-2 antigens was detected on the surface of JAR cells at any time after the treatment with AzaC. This confirms that DNA hypomethylation may not be sufficient to cause gene expression (23).

The findings presented here may have interesting implications for the physiology of trophoblast and sperm. Trophoblast, the histotype of origin of CCs, and sperm are among the few types of nucleated cells in the body that do not express HLA class I (5–7). A tight mechanism of regulation like DNA methylation may be necessary in trophoblast as well as in CC lines to prevent the expression of these genes, since expression may lead to an immune response of the mother against the fetus. On the other hand, sperm DNA efficiently transfects genes for HLA class I, thus indicating that these genes are regulated differently in sperm and in CC cells. Sperm DNA does not transfect the gene for Leu-1. By analogy with the results obtained with the CC lines, methylation is the likely cause of this finding.

We thank Dr. Charles Hsu for his help with the transfection, Mark Upton for technical help, and Linda Lloyd for excellent secretarial help. This work was supported in part by National Institutes of Health Grant CA-42509 and by the Italian Association for Cancer Research.