Differentiation of mouse embryonic stem cells and their hybrids during embryoid body formation

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Abstract

We studied the karyotypes of three hybrid clones of mouse embryonic stem cells and murine splenocytes (two having near diploid and one having near tetraploid chromosome numbers) and the characteristics of their differentiation during the formation of embryoid bodies. The X chromosome originating from embryonic stem cells may be lost in hybrids with a near diploid chromosome number and reprogramming of the "somatic" X may occur. The morphological data we obtained using light and electron microscopy revealed a correlation between the karyotype constitution of hybrid cells and their differentiation during the formation of embryoid bodies. At the beginning of development, the embryoid bodies derived from hybrid cells already showed an advanced degree of differentiation. The production of significant quantities of cartilage was typical for hybrid cells with near tetraploid chromosome numbers. The hybrid cells showed restricted pluripotent capacity and were already committed when they started to differentiate into embryoid bodies.

Key words: mouse ES-somatic cell hybrids, karyotype, pluripotency, differentiation, embryoid bodies.

Received: May 2, 2000; accepted: July 3, 2000.

Introduction

Hybrids between pluripotent cells such as murine embryonic carcinoma cells (EC) and somatic cells (fibroblasts, splenocytes, lymphocytes etc.) can be used to study the differentiation of hybrid cells. Chromosome sets in hybrid cells can influence phenotype and differentiation capacity, giving new characteristics to the cells (Kerkis and Zhdanova, 1992; Takagi, 1997). Hybrids of EC cells with differentiated somatic cells have the phenotype of EC pluripotent cells and are able to differentiate into various tissues, derived from embryonic germ layers, and express some embryonic antigens (Miller and Ruddle, 1976; 1977; Litwack, and Croce, 1979; Andrews and Goodfellow, 1980; Roussel et al., 1983; Takagi et al., 1983; van der Kamp et al., 1984; Subramanian, 1989).

The pluripotency of cells has been evaluated by subcutaneous injection into syngeneic mice, resulting in the formation of teratocarcinomas. During differentiation not all hybrid clones produce the same pattern of cellular diversity (Miller and Ruddle, 1976). The restriction of the pluripotency of some hybrid clones may depend on their karyotype, but using an animal model it is difficult to determine the influence of karyotype because the differentiation of cells occurs under the developmental control of the parental organism.

Alternatively, the pluripotency of the cells can be examined by their capacity to form embryoid bodies (Ebs), which have similar characteristics to the initial stages of embryo development. This model has been shown to be useful in studies of cell differentiation (for reviews see: Pedersen, 1994; Keller, 1995). Such cells permit the study of the differentiation of pluripotent cells in vitro without the developmental influence of the animal environment. Under standardized culture conditions the observed differentiation can be attributed to the pluripotent cell genome.

Mouse embryonic stem cells (ES) are another type of pluripotent cell, which can be obtained from the inner cell mass of a normal embryo (Martin, 1981; Evans and Kaufman, 1981). Embryonic stem cells have been fused with differentiated mouse somatic cells (Matveeva et al., 1996) and human microcells (Tomizuka et al., 1997). The resulting hybrids had pluripotent characteristics in culture and could be used for the construction of transgenic (Tomizuka et al., 1997) and chimeric animals (Matveeva et al., 1998). Such hybrid cells can serve as a model for the
study of the putative influence of the supplemental somatic chromosomes and other factors on differentiation.

In the study presented in this paper we used three mouse hybrid clones obtained by fusion of embryonic stem cells with splenocytes. We examined the karyotypes of these clones and investigated the influence of the karyotypes on the differentiation of these cells through the formation of embryonic bodies.

Materials and Methods

Embryonic stem cells and their hybrids

HM-1 cells, deficient in hypoxanthine phosphoribosyltransferase (HPRT), were derived from HPRT-deficient strain 129 mice (Magin et al., 1992) and characterized as highly pluripotent (Magin et al., 1992; Selfridge et al. 1992). The HESS-1, HESS-2 and HESS-3 hybrid cells were isolated by the fusion of HM-1 cells with murine splenocytes of DD/c female, and characterized as pluripotent and HPRT positive by Matveeva et al. (1996, 1998).

We maintained the HM-1, HESS-1, HESS-2, HESS-3 cells in an undifferentiated state using a feeder layer of murine embryonic fibroblasts inactivated by mitomycin C (10 µg/mL for 3 h). For cell cultivation without a feeder layer, the medium described below was supplemented with 10³ units/mL of murine leukemia inhibitory factor (mLIF, Sigma) and Petri dishes were previously treated with an aqueous solution of 0.1% gelatin.

The HM-1 and hybrid cells were cultivated in the alpha-modification of Eagle’s medium (αMEM, Sigma) supplemented with 3.5g/L D(+)-glucose (Sigma), 10% (v/v) bovine fetal serum, 10⁻⁴ M β-mercaptoethanol, 50 U/mL penicillin and 50 µg/mL streptomycin. For hybrid cell cultivation αMEM was supplemented with 100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine (HAT, Gibco), and the cells were passaged every 3 days onto a fresh feeder layer. Cultures were maintained in a humid atmosphere containing 5% CO₂ at 37 °C.

For selection by 6-thioguanine, hybrid cells were plated in complete Eagles culture medium supplemented with 10⁷ units/mL mLIF and 30 µg/mL 6-thioguanine on gelatinized 60 mm dishes (3600-3800 cells/dish), the colonies being examined after 8-10 days of cultivation.

Embryoid body formation

Embryoid bodies were obtained by cultivation of ES cells and their hybrids in suspension according to the protocol of Robertson (1987), using the same medium as that for undifferentiated cell culture, excluding mLIF and β-mercaptoethanol. Embryonic stem and hybrid cells were trypsinized and the suspension left in a culture flask in the CO₂ incubator for 15 min to eliminate fibroblasts, after which the suspension was transferred to another flask and incubated overnight. On the following day cell aggregates were separated by shaking, re-suspended in culture medium and placed into bacteriological dishes, the medium being changed every 2-3 days.

Alkaline phosphatase activity and cytogenetic analysis

The hybrid and HM-1 cells growing on microscope slides with feeder cells were washed in 0.1 M PBS, pH 7.4 and fixed with 4% paraformaldehyde in PBS for 15 min. Standard histochemical techniques were used for the detection of alkaline phosphatase (AP) activity in the undifferentiated cells (Talbot et al., 1993).

For chromosome studies all cell lines were treated according to Hogan et al., 1994 and trypsin-Giemsa banding were obtained by the method of Seabright 1971. At least 250 different metaphases from each line were analyzed. Embryoid bodies were pre-treated with type III collagenase (Sigma).

Optical and electron microscopy studies of EBs

For optical microscopy embryoid bodies were fixed with 4% paraformaldehyde in 0.2 M PBS for one hour at room temperature and maintained at 4 °C overnight before being embedded in paraffin and cut into 3-5 µ sections which were then Gomori stained (Behmer et al., 1976).

For electron microscopy the embryoid bodies were fixed in a mixture of 2.5% gluteraldehyde, 4% paraformaldehyde and 0.1 mM CaCl₂ in 0.1M cacodylate buffer (pH 7.4) for 1 h. Post-fixation was performed with 1% osmium tetroxide (OsO₄) in the same buffer for 30 min, followed by embedding in Epon 812. After polymerization, sections were cut with a Reichert’s Ultracut ultramicrotome using a diamond knife. The sections were treated with an aqueous solution of uranyl acetate and lead citrate to enhance contrast (Reynolds, 1963). The samples were analyzed using a Zeiss IN 900 electron microscope.

Results

Morphologic pattern of the hybrid cell cultures

Colonies formed by the HESS-2 and HESS-3 hybrid cells were similar to HM-1 cells, being composed of many small, round and juxtaposed cells. HESS-1 cells were less similar to the parental HM-1 line and their colonies formed on a fibroblast layer were heterogeneous and consisted of large, round cells. On the top of the colony there were some small cells similar to HM-1 cells.
Cytogenetic analysis

HM-1 cells were stable and had a normal diploid chromosome number of 2n = 40 (38A + XY), without any aneuploidy or structural alterations up to 30 passages in culture.

Analysis of HESS-1 cells after 25 passages showed that this hybrid was heterogeneous, with chromosome numbers from 2n = 40 up to 80, with a modal number of 71 chromosomes. Different patterns of sexual chromosomes were observed, the XXXY karyotype being frequently seen and the XXY karyotype rarely observed. The karyotypes with 41-43 chromosomes included trisomies for chromosomes 1, 11, 14 and 16.

Karyotype analysis of HESS-2 cells after 38 passages showed a predominance of cells with 42 chromosomes. Almost all metaphases had a pair of XY sex chromosomes. Among 47 metaphase spreads only seven (15%) had two X chromosomes, while 50% showed a chromosome fragment, probably derived from chromosome 5. Trisomy of chromosomes 1, 11, 12 or 16 was observed, Figure 1 showing the XY karyotype with trisomy 11 and the chromosome fragment.

The modal chromosome number of HESS-3 cells after 39 passages was 41, including only one X chromosome. Cells with XXY or XX sex chromosomes were not observed, although trisomies for chromosomes 1, 11, 15 or 17 were detected (Figure 2).

Cytogenetic analysis of embryoid bodies showed that the karyotypes of these bodies on the 7th day of their formation had almost the same modal number as the hybrid clones in cell culture (Table 1).

**Table 1 - Frequency of different chromosome numbers in 7-day embryoid bodies derived from HESS-1, HESS-2 and HESS-3 hybrid cells.**

<table>
<thead>
<tr>
<th>Chromosome number</th>
<th>Number of metaphases analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HESS-1</td>
</tr>
<tr>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>41</td>
<td>2 (4.2)(^1)</td>
</tr>
<tr>
<td>42</td>
<td>5 (10.4)</td>
</tr>
<tr>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>64</td>
<td>1 (2.1)</td>
</tr>
<tr>
<td>68</td>
<td>1 (2.1)</td>
</tr>
<tr>
<td>69</td>
<td>6 (12.5)</td>
</tr>
<tr>
<td>70</td>
<td>2 (4.2)</td>
</tr>
<tr>
<td>71</td>
<td>4 (8.3)</td>
</tr>
<tr>
<td>72</td>
<td>11 (22.9)</td>
</tr>
<tr>
<td>73</td>
<td>6 (12.5)</td>
</tr>
<tr>
<td>74</td>
<td>6 (12.5)</td>
</tr>
<tr>
<td>75</td>
<td>3 (6.2)</td>
</tr>
<tr>
<td>77</td>
<td>1 (2.1)</td>
</tr>
</tbody>
</table>

\(^1\)Percentage in parenthesis.

**Figure 1** - GTG-banded karyotype of a HESS-2 cell with trisomy of chromosome 11 and a structural rearrangement (R) (Passage 38).

**Figure 2** - GTG-banded karyotype of a HESS-3 cell with trisomy of chromosome 1 and 17 (Passage 39).
Origin of the X chromosome in the hybrid cell

Most HESS-2 and HESS-3 cells had only one X chromosome. To investigate the origin of this chromosome cells were treated with 6-thioguanine, a chemical which allows the selection of HPRT-deficient cells (Verma and Babu, 1995). HESS-3 cells were unable to grow in the presence of 6-thioguanine but were able to grow in the selective HAT medium (Table 2). This was a predictable result, since before selection with 6-thioguanine the HESS-3 cells could not have lost the ‘somatic’ X chromosome due to the pre-cultivation in HAT medium. Therefore the frequency of cells with only one chromosome X of ‘embryonic origin’ in the HESS-3 hybrid cells was less than 2.6 x 10^-4.

When the HESS-2 cells were repeatedly cultivated in the presence of HAT approximately 15% of the cells had two X chromosomes, as described above. It is conceivable that in the absence of selective pressure these cells lost one of their X chromosomes, i.e. the ‘somatic origin’ X chromosome could be preferentially lost, as the chromosome of the more differentiated partner of cell fusion (Ringertz and Savage, 1976). We cultivated HESS-2 cells for 27 days (11 passages) without HAT and then subjected them to selection with 6-thioguanine (Table 2). None of the colonies of the hybrid cells grew in the culture medium with 6-thioguanine (a frequency below 2.7 x 10^-4), whereas HAT-resistant colonies had a frequency of about 3 x 10^-5. These results show that in the absence of selective pressure (during about 50 cell divisions) the X chromosome of somatic origin (HPRT+) was not lost, instead the X chromosome originating from the ES cells (HPRT+).

Alkaline phosphatase (AP) activity

Table 3 shows the percentage of AP positive cells in the HM-1 and hybrid cells. The largest numbers of AP positive cells (about 94%, Figure 3a) were found in HM-1 cells but the HESS-3 line also had a large percentage (higher than 90%) of AP positive cells. HESS-2 had a smaller number of AP positive cells (about 56%, Figure 3b) and the HESS-1 line the smallest number of AP positive cells (about 14%). In general, among the heterogeneous cells of the HESS-1 line only small cells were AP positive, whereas larger cells were weakly stained (Figure 3c).

Optical and electron microscopy of embryoid bodies

Comparative analysis of sections of EBs revealed a variety of differentiated cells in the hybrids and the HM-1 line. In the EBs originating from HM-1 cells, endodermal, cartilage-like and blood-like cells were marked positively at day 9 (Figure 4a). In EBs derived from HESS-2 and HESS-3 lines the formation of a large amount of cartilage matrix was observed in different parts of the EBs sections (Figure 4b). The inner part of the EBs showed a larger amount of cartilage matrix in sections obtained from HESS-1 cells (Figure 4c). On the periphery of the bodies a layer of the endodermal cells and islands of blood cells were also observed (Figures 4b and 4c).

Electron microscopy of the EBs revealed distinctive differences in the EBs formed by hybrids and by HM-1 cells. At day 3, EBs derived from HM-1 had an outer layer of endodermal cells. These cells were at the beginning of their differentiation since they possessed few microvilli and few cytoplasmic organelles (Figure 5a). The central part of the EBs consisted of round undifferentiated cells with a large nuclei and cytoplasm with a small number of organelles (Figure 5b). On day 9, the outer layer of HM-1 derived EBs showed epithelium-like cells with numerous microvilli. These cells had junction structures of the desmosome type and Reichert’s membrane was well developed and the cystic cavity could be seen. In the inner part of the bodies muscular-like cells, adipose-like and haematopoietic stem-like cells at various stages of differentiation (Figure 5c,g) were also found. On day 9, the HM-1 derived EBs showed the characteristic rhythmic contractions of heart muscle cells.

On day 3, EBs derived from hybrids presented an advanced degree of differentiation. Embryoid bodies derived from HESS-2 and HESS-3 lines had an endodermal layer.

Table II - Comparison of plating efficiency of the hybrid cells in the presence of 6-thioguanine and HAT (100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine).

<table>
<thead>
<tr>
<th>Hybrid</th>
<th>Passage number</th>
<th>Number of cells</th>
<th>6-thioguanine resistant colonies</th>
<th>HAT-resistant colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>HESS-3</td>
<td>39</td>
<td>3800</td>
<td>0 (2.6 x 10^-4)</td>
<td>52 (1.4 x 10^-5)</td>
</tr>
<tr>
<td>HESS-2*</td>
<td>47</td>
<td>3600</td>
<td>0 (2.8 x 10^-4)</td>
<td>115 (3.2 x 10^-5)</td>
</tr>
</tbody>
</table>

1Frequency.
*These cells were pre-cultivated in the absence of HAT for 27 days (11 passages).

Table III - Percentage of alkaline phosphatase positive cells in undifferentiated cultures of HM-1 cells and their hybrids.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Total number of cells</th>
<th>Number of AP-positive cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>HM-1</td>
<td>1106</td>
<td>950 (94.4)%</td>
</tr>
<tr>
<td>HESS-1</td>
<td>11484</td>
<td>1597 (13.9)%</td>
</tr>
<tr>
<td>HESS-2</td>
<td>2320</td>
<td>1305 (56.3)%</td>
</tr>
</tbody>
</table>

1Percentage in parenthesis.
formed by cells already possessing multiple microvilli and an organelle-enriched cytoplasm. The start of Reichert’s membrane formation was initiate and cells resembling embryonic fibroblasts could be seen under this membrane (Figure 6a). The central part of individual EBs contained morphologically undifferentiated cells. At a comparable stage of cultivation, EBs derived from HESS-1 hybrid cells were not very compact and the large spherical cells on their outer surface probably had near tetraploid chromosome...
numbers. These cells had a cytoplasm rich in organelles, mainly granular endoplasmatic reticulum (Figure 6b).

From day 9 to 18, the outer layer of endodermal cells had a greater number of microvilli and their cytoplasms were filled with endoplasmatic reticulum. The cystic cavities were well formed (Figure 6c) and areas resembling blood cell islands could be seen at the center of the EBs (Figure 6d). An important characteristic of differentiated hybrid cells was the production of a large number of polysaccharide granules and a lot of extracellular collagen secreted by chondroblasts or fibroblast-like cells (Figure 6e and 6f).

Embryoid bodies derived from hybrids lacked cells of the muscular type. To investigate whether these cells had the capacity to differentiate into muscle cell, we allowed the EBs to develop up to 30 days. Only a few HESS-2 derived EBs presented muscular contractions on the 10th to 15th day, the other hybrids not showing such contractions.

**Discussion**

We attempted to evaluate the influence of near diploid and near tetraploid karyotypes on the in vitro differentiation of hybrid cells obtained by fusion of somatic and
embryonic genomes. If karyotype is influential, its effects would appear at the initial stages of embryo formation which is very sensitive and important for normal development. For this reason we chose Eb's as a model of early development, a choice which permitted us to study differentiation without any influence of the parental organism.

It is noteworthy that the intraspecific hybrids between the pluripotent and somatic cells usually have a near tetraploid chromosome number (Takagi, 1997). The hybrid cells used in our study were near diploid (HESS-2 and HESS-3) and near tetraploid (HESS-1) and our chromosome analysis showed different trisomies, trisomies of chromosomes 1 and 11 being found in near diploid hybrids. These trisomies are probably typical of pluripotent cells, and have previously been described in the MESC embryonic cell line (Crolla et al., 1990) and in embryonic carcinoma cells (McBurney and Rogers, 1982).

We found that the sex chromosome constitution in the HESS-2 line was predominantly XY, while in the HESS-3 line it was XO. This observation was unexpected since in the hybrid cells two X chromosomes should stay together because one X had the HPRT gene that was maintained by cultivation in HAT, while the other X belonged to the pluripotent embryonic cells. Indeed, it has been demonstrated by Ringertz and Savage (1976) that hybrids lose the chromosomes originating from differentiated, more slowly dividing, cells. Thus our results raised the question of the origin of the X chromosome in the hybrid cells, and to answer this question we used 6-thioguanine selection. In HESS-2 and HESS-3 lines, the segregated X chromosome was demonstrated to be of embryonic origin. Therefore, the segregation of the X chromosome of the more differentiated fusion partner during non-selective cultivation is not a rule in these hybrids.

During embryoid body formation the selection of cells with a diploid (2n = 40) karyotype is possible, but we did not detect selection for cells with a given chromosome number since the karyotypes of 7 day hybrid-derived embryoid bodies (EBs) had maintained the same modal chromosome number as the cells in monolayer culture.

In culture, HESS-1, HESS-2 and HESS-3 lines had, like other hybrids (q.v. review in Takagi, 1997), the phenotype of pluripotent cells, the AP-reaction being considered as a marker of pluripotency (Resnick et al., 1992; Talbot et al., 1993). We found the highest number of AP-positive cells in the HESS-3 and HESS-2 cell lines, which most resembled the parental HM-1 line in morphological characteristics. The HESS-1 line was heterogeneous for the AP-reaction, HESS-1 AP-positive cells having near diploid chromosome numbers and AP-negative cells near tetraploid chromosome numbers.

In our experiments, hybrids showed the capacity to form EBs in vitro, even at late passages. The EBs formed by the hybrid cells could be considered as complex as those derived from the HM-1 line and the cystic-type EBs formed by pluripotent cells (Martin and Evans, 1975; van der Kamp et al., 1984; Doetschman et al., 1985; Pease et al., 1990).

The formation of blood cell islands, similar to the yolk sac, has been described in EBs derived from different ES cell lines (Doetschman et al., 1985; Hollands, 1988; Nichols et al., 1990; Chen, 1992; Bautch et al., 1996). We also observed haematopoietic-like cells in the EBs derived from hybrids and HM-1 cells. The formation of these cell types was related to the presence of visceral endoderm-like cells which we detected using electron microscopy. These cells possessed microvilli on their surface, cytoplasmatic vesicles and gap junction-like structures. Bautch et al. (1996) observed the precursors of haematopoietic cells that were localized in the center of EBs, but they migrated to the periphery as soon as they became differentiated. Our electron microscopy and histochemical analyses revealed cells with a high cytoplasm:nucleus ratio in the central area of EBs formed by the hybrids. These cells may be considered haematopoietic-like precursors.

Cells resembling skeletal and smooth muscle were seen during differentiation of the HM-1 line. The presence of the mesodermal cells showed that EBs obtained from this line follow the typical embryoid body pattern of development (Doetschman et al., 1985; Nichols et al., 1990; Bautch et al., 1996). The hybrid cells did not show this type of differentiation in EBs at the initial stages of development, suggesting restricted pluripotency.

Cells of ectodermal origin (e.g. nerve cells) were not identified in EBs derived from HM-1 cells and hybrids. The absence of nerve cells might be explained because cell differentiation took place in suspension, while nerve cells require a solid substrate and specific inducers for differentiation (Bain et al., 1995; Fraichard et al., 1995).

Studies of the influence of the karyotype on ES cell pluripotency have demonstrated that the normal karyotype may be a prerequisite for the efficient contribution of these cells to the germ line in transgenic and chimeric animals and for their ability to differentiate in vitro into a wide spectrum of cell types (Papaioannou et al., 1978; McBurney, Rogers, 1982; Pease et al., 1990; Bronson et al., 1995; Liu et al., 1997; Suzuki et al., 1997). Our data shows that the ‘embryonic’ X chromosome may be lost in pluripotent hybrids, but reprogramming of the ‘somatic’ X chromosome may still occur, thus allowing restricted pluripotency. Near diploid and near tetraploid hybrids did not differ as to their pluripotency, but appeared, however, to be restricted as compared to normal ES cells.

Acknowledgments

We are thank Dr. O. Serov for the HM-1 and hybrid cells, Dr. Ekkehard Hansen for helpful suggestions and Ms M.A. da Silva Carvalho Dutra for taking the photographs. Our special gratitude to Dr. Antonio Cordeiro for his
References


