Effect of Bredinin on Early Embryonic Development in Mice

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Bredinin, an immunosuppressive agent, had a teratogenic effect and a decreasing effect in litter size in mice and in rats. We investigated the direct effect of bredinin on the development of mouse preimplantation embryos in vitro. Bredinin strongly inhibited the first differentiation step from the morula to the blastocyst stage, whereas the steps from the two-cell embryo to the morula stage were not inhibited. At 10^{-5} or 10^{-4} M of bredinin, only one or none of the morula embryos developed to a blastocyst after a 48 h culture; while approximately 90% of the embryos developed to blastocysts in the absence of bredinin. To determine whether the bredinin inhibition was reversible, the morula embryos were cultured with 10^{-5} M of bredinin for 48 h. The embryos were transferred to bredinin-free culture conditions, and their development was observed. The inhibitory effect of bredinin was reversible. The expansion of zona-free blastocyst was also inhibited by bredinin. When blastocyst embryos were cultured in the presence of bredinin (10^{-5} M), the mean size of the embryos was reduced to half the initial size. In addition, the steps of hatching from the zona pellucida and attaching to the substratum were significantly inhibited by bredinin. Trophoblast outgrowth was completely inhibited by 10^{-6} M bredinin; however, the area of trophoblast outgrowth was expanded to 18-fold the initial size after a 96 h culture in the absence of bredinin. Thus, the decrease in the number of offspring is due to the direct effect of bredinin on mouse embryos.

Keywords bredinin; embryonic development; in vitro; stage-specific inhibition

Introduction

Bredinin (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate) is an immunosuppressive agent isolated from the culture supernatant of Eupenicillium brefeldianum M-2166.1,2) It has been reported that many carcinostatic or immunosuppressive agents have mutagenic, teratogenic and cytotoxic effects. Especially on the teratogenic effects of 6-mercaptopurine and azathiopurine (imuran), there are many reports available.3–10) Kobayashi et al. reported that bredinin had a teratogenic effect in mice.11) We also found the same effect of bredinin in rats.5,9) In addition, we showed that the number of offspring was reduced by the administration of bredinin into pregnant mice and rats.

Preimplantation mouse embryos cultured in vitro are quick, reproducible and highly sensitive for assessing the effect of toxic substances on embryonic development.10,11) By contrast, the in vitro culture of preimplantation rat embryo has not been described. Recently, the inhibitory effects of purines and pyrimidines on the in vitro development of preimplantation mouse embryos were studied.12,13)

Bredinin inhibited the proliferation of mouse embryo cells in vitro.9) This inhibitory effect against embryonic cells was diminished by the addition of guanosine 5'-monophosphate (GMP) to the culture medium. By contrast, the inhibition was not altered by the addition of inosine or inosine mono-phosphate (IMP). We concluded that bredinin is an inhibitor of enzyme in the pathway IMP to GMP.

We examined the direct effect of bredinin on the development of mouse embryos in vitro to clarify the teratogenic effect and decreasing effect in the litter size of bredinin. Furthermore, we also examined the recovery effect of guanine (GN) derivatives on embryonic development inhibited by bredinin.

Materials and Methods

Collection and Culture of Embryos in Vitro To induce superovulation, female ICR mice aged 8–10 weeks (Clea Japan Inc., Japan) were intra-peritoneally treated with 5 IU of pregnant mare's serum gonadotropin (PMSG, Teikoku-zoki) and 48 h later with 5 IU of human chorionic gonadotropin (HCG, Teikoku-zoki). The females were caged with ICR males after HCG injection. The females with vaginal plugs were isolated and housed for a further 24–96 h. Embryos were collected from superovulated mice by flushing the reproductive tracts with a small volume of culture medium at given times after HCG injection: 2-cell embryos, at 24 h; morulae, at 72 h; blastocysts, and at 96 h as previously reported.14) The term morula generally refers to a compact aggregated of blastomeres (Fig. 1b), since morula is all stages in development from compacted 8-cells to the 16- to 32-cell stage just prior to blastocoele formation. The embryos which form a blastocoele (Fig. 1c) are considered as the blastocyst stage. Zona-free blastocysts were obtained by the incubation of blastocysts for one min in 0.1 M glycine–HCl buffered saline (pH 3.0) containing 0.4% bovine serum albumin (BSA) at room temperature. The embryos were pooled in watch glass and washed three times in phosphate buffered saline (PBS). A group of 10–20 embryos was then added to each 24-well tissue culture multi-dish (Linbro). The dishes were incubated in the presence or absence of bredinin (kindly gifted from Toyo-Jyoz Co., Ltd.) in culture medium for given periods at 37°C under a gas phase of 5% CO2 in air. The culture medium was Ham's F-12 (Nissui) supplemented with 10% calf serum (Flow Laboratories Inc.) At every 12 h during the culture period, developing embryos were observed under a phase contrast microscope (Nikon, Type MD). For hatching assay, blastocysts were considered hatched only when fully escaped from the zona pellucida (Fig. 1d). For blastocyst expansion assay and trophoblast outgrowth, cultured embryos were photographed through a Nikon phase contrast microscope. The size of the embryo and the area of trophoblast outgrowth were expressed in square millimeter (major axis × minor axis) 100 magnification.

For recovery assay, GN, guanosine (GS), guanosine 5'-monophosphate (GMP) and dibutyryl-guanosine 3':5'-cyclic monophosphate (dgGMP) were obtained from Sigma. Thirty to forty zona-free blastocysts were cultured with bredinin (10^{-7} M) in the presence or absence of GN derivatives (5 x 10^{-7} M) for 96 h. At the end of the culture period, the trophoblast outgrowth area was measured as above. The data are expressed in the mean ± standard error of all embryos.

Statistical Analysis Student's t-test was used for comparison between treated and non-treated embryos (Tables IV, VI and Fig. 3). The chi-square analysis was used for statistical analysis of the other Table.

Results

To determine the direct effect of bredinin on the develop-
opment of mouse embryos, embryos at various stages were used. Approximately 75% of 2-cell embryos developed to morulae after a 48 h culture in the presence or absence of bredinin. Thus, bredinin exerted no effect on the embryonic development from the 2-cell stage to morula stage (Table I).

Table II shows the development of morula-stage embryos after a 48 h culture. The embryos possessing the blastocyst were considered as at the blastocyst stage (Fig. 1c). Eighty nine percent of the embryos (39/44) developed to blastocysts in the absence of bredinin (control). On the
other hand, only 47% of the embryos (20/43) developed to the blastocyst stage in the presence of bredinin (10^{-7}M). At a higher concentration of bredinin (10^{-6}M), only one embryo grew to a blastocyst but no embryos developed at 10^{-5}M of bredinin (Table II). When embryos were pretreated with 10^{-5}M of bredinin for 48 h, washed with fresh medium, and then cultured in the absence of bredinin, they started development (data not shown).

Table III shows that bredinin caused a significant decrease in the percentage of hatched embryos from the zona pellucida. At 10^{-7}M of bredinin, 30% (9/30) of the embryos were hatched while 75% (24/32) of the embryos were hatched in the control medium after in vitro 48 h culture. But no complete inhibition was observed, even at 10^{-5}M of bredinin.

Very little was known about the hatching. On the basis of a hypothesis that the zona pellucida ruptured because of elevated internal hydrostatic pressure by the accumulation of blastocoel fluid, we measured the size of the embryos. Bredinin caused a marked decrease in blastocyst expansion. When blastocysts were cultured for 48 h in the control medium the mean size of embryos doubled the initial value (initially, 68.5 \times 10^4 mm²; after 48 h culture, 132.2 \times 10^4 mm²). On the contrary, when embryos were cultured in the presence of bredinin (10^{-6}M), the mean size of the embryos was reduced to half of the initial size (initially, 66.6 \times 10^4 mm²; after a 48 h culture, 27.0 \times 10^4 mm²) (Table IV). Thus, bredinin completely blocked the expansion of the embryos.

To assess the attachment to the substratum, gentle vibration was applied to the culture dish. We considered the embryos as attached when they were not dislodged by this procedure. All embryos attached to the substratum after a 72 h culture in the absence of bredinin. Bredinin slightly inhibited the attachment of zona-free blastocysts. Even at the highest concentration tested (10^{-5}M), 70% (24/34) of zona-free blastocysts attached to the culture dish after a 72 h culture, and that attachment was retarded by bredinin (Table V).

The rate of trophoblast outgrowth was also inhibited by bredinin, as is clear in Fig. 2. Whereas all zona-free blastocysts outgrew on the substratum within 72 h in the control medium, approximately 50% of blastocysts had trophoblast outgrowth after a 96 h culture in the presence of 10^{-5}M of bredinin. Table VI shows trophoblast grew to 18-fold the initial size in the absence of bredinin after a 96 h culture. The trophoblasts outgrowth were completely

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**Table III. Inhibitory Effect of Bredinin on the Hatching of Blastocyst**

<table>
<thead>
<tr>
<th>Bredinin (M)</th>
<th>No. of blastocysts</th>
<th>No. of hatched cells</th>
<th>Hatching (%)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>32</td>
<td>24</td>
<td>75.0</td>
<td>0</td>
</tr>
<tr>
<td>10^{-7}</td>
<td>50</td>
<td>9</td>
<td>30.0(^a)</td>
<td>60.0</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>36</td>
<td>7</td>
<td>19.4(^a)</td>
<td>74.1</td>
</tr>
<tr>
<td>10^{-5}</td>
<td>31</td>
<td>3</td>
<td>9.7(^a)</td>
<td>87.1</td>
</tr>
</tbody>
</table>

The embryos (blastocysts) were recovered from the uterine horns 96 h after HCG injection and cultured in Ham’s F-12 with 10% calf serum in the presence or absence of bredinin in a Linbro plastic multi-dish for 48 h at 37°C. *a* Significantly different from the control value (0 mol bredinin) (p < 0.01).

**Table IV. Inhibitory Effect of Bredinin on the Expansion of Blastocyst**

<table>
<thead>
<tr>
<th>Bredinin (M)</th>
<th>No. of blastocysts</th>
<th>Size of blastocyst (mean ± S.E.)</th>
<th>Cultivation time (h)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33</td>
<td>68.5 ± 1.9</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>10^{-6}</td>
<td>33</td>
<td>66.6 ± 1.9</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>10^{-5}</td>
<td>34</td>
<td>69.4 ± 1.9</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

The blastocysts were prepared as described in Materials and Methods, and cultured in Ham’s F-12 containing 10% calf serum with or without bredinin in a Linbro plastic multi-dish at 37°C for indicated periods in 5% CO₂-air. The embryos were photographed through a Nikon phase contrast microscope. The size of embryos were measured on the photographs and expressed in square millimeter (major axis × minor axis) at 100 magnification. *a* Significantly different from the control value (0 mol bredinin) (p < 0.01).

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**Table V. Inhibitory Effect of Bredinin on the Attachment of Zona-Free Blastocyst**

<table>
<thead>
<tr>
<th>Bredinin (M)</th>
<th>No. of zona-free blastocyst</th>
<th>No. of attached embryo (%)</th>
<th>Cultivation time (h)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33</td>
<td>4 (12.1)</td>
<td>48</td>
<td>72</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>33</td>
<td>5 (15.2)</td>
<td>22 (66.7)(^n)</td>
<td>24</td>
</tr>
</tbody>
</table>

The zona-free blastocysts were prepared as described in Materials and Methods, and cultured in Ham’s F-12 containing 10% calf serum with or without bredinin in a Linbro plastic multi-dish at 37°C for the indicated periods. The attachment of embryo onto the culture dish was examined by phase contrast microscopy. *n* Significantly different from the control value (0 mol bredinin) (p < 0.01).

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**Table VI. Inhibitory Effect of Bredinin on Trophoblast Outgrowth**

<table>
<thead>
<tr>
<th>Bredinin (M)</th>
<th>No. of trophoblast outgrowth (mean ± S.E.)</th>
<th>Area of trophoblast outgrowth (mean ± S.E.)</th>
<th>Cultivation time (h)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>33</td>
<td>68.5 ± 1.9</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>10^{-6}</td>
<td>33</td>
<td>66.6 ± 1.9</td>
<td>93.2 ± 13.2(^n)</td>
<td>60.0 ± 6.3(^n)</td>
</tr>
</tbody>
</table>

The blastocysts were prepared as described in Materials and Methods, and cultured in Ham’s F-12 with 10% calf serum with or without bredinin in a Linbro plastic multi-dish at 37°C for indicated periods in 5% CO₂-air. The embryos were photographed through a Nikon phase contrast microscope. The area of trophoblast outgrowth was measured on the photographs and expressed in square millimeter (major axis × minor axis) at 100 magnification. *n* Significantly different from the control value (0 mol bredinin) (p < 0.01).
inhibited by $10^{-6}$ M of bredinin.

Figure 3 illustrates the recovery effect of GN derivatives on trophoblast outgrowth inhibited by bredinin. The area of trophoblast outgrowth in the presence of $10^{-7}$ M of bredinin was about half that of control (bredinin-free media). The addition of five molar excess ($5 \times 10^{-7}$ M) of GN derivatives (GN, GS, GMP and cGMP) produced a recovery effect on bredinin-inhibited trophoblast outgrowth. Especially the addition of GMP or cGMP to the culture medium in the presence of bredinin caused a marked recovery. The mean areas of trophoblast outgrowth in the presence of GMP or cGMP were 120% of the control area (in the case of bredinin-free media). But these enlarged areas were not significantly different ($p > 0.05$) from that observed in the absence of bredinin.

Discussion

It has been reported that bredinin has a teratogenic effect.\(^7\) When [\(^{14}\)C]bredinin was orally administered to pregnant rats, radioactivity was found in the fetuses.\(^6\) Those findings suggested a possibility that the teratogenic effect of bredinin is due to its direct inhibitory effect on embryonic development. Preimplantation mouse embryos cultured in vitro provide a quick and highly sensitive means for assessing the toxic effect of substances on embryonic development.\(^10,11\) In the present study we examined the direct effect of bredinin on embryonic development in mice in vitro. Bredinin especially showed an inhibitory action on the development of mouse embryo from the morula to the blastocyst stage and trophoblast outgrowth at $10^{-6}$ M. In contrast, the steps from two-cell to morula were not inhibited at all at $10^{-5}$ M. Thus, the susceptibility to bredinin varied in the developmental stages.

Recently, the direct effects of purines on the in vitro development of pre-implantation mouse embryos were studied.\(^12\) Hypoxanthine, adenosine and inosine showed reversible inhibition on the development from the 2-cell to the morula stage at $3 \times 10^{-6}$ M, but GS did not show an inhibitory effect at the same concentration. In this study, bredinin showed the inhibitory effect on embryonic development in vitro at a lower concentration than purines showed. Accordingly, this inhibition is thought to be due to the elevation of the osmotic pressure by the addition of bredinin.

It was also reported that bredinin had a cytotoxic effect on LS178Y cells. The inhibitory effect of bredinin was recovered by the addition of GS and GMP.\(^2\) Accordingly, a possible explanation for the blocking mechanism of bredinin is thought to be that the pathway from xanthosine 5'-monophosphate (XMP) to GMP is blocked by bredinin. Furthermore, some purines other than GS inhibited the development of mouse embryo in vitro.\(^12\) We tried to determine that the inhibitory effect of bredinin on trophoblast outgrowth could be prevented by the addition of GN derivatives in vitro. Although GMP was not expected to enter the cells and of itself prevented bredinin-induced inhibition, it prevented the inhibition probably because it cleaved to GS and/or GN, both able to enter the cells.

The steps from the 2-cell to the morula stage were not inhibited by bredinin. This phenomenon was also seen in the case of [\(^{3}\)H]thymidine.\(^3\) We assume that embryos possess a larger nucleoside pool in their early developmental stage than in their blastocyst stage. When 2-cell embryos were cultured in PBS, the 2-cell embryos could develop to morulae even in protein- and nutrition-free media but trophoblast outgrowth was not observed in protein-free media (data not shown). We conclude that mouse embryos at an early stage of development possess a larger nucleoside pool than morula and/or blastocyst stage.

References

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