THALIDOMIDE-INDUCED SUPPRESSION OF EMBRYO FIBROBLAST PROLIFERATION REQUIRES CYP1A1-MEDIATED ACTIVATION

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ABSTRACT:

An enzyme involved in the metabolic activation of thalidomide has been investigated using embryo fibroblast proliferation as a marker. Thalidomide (30 μM) induced suppression of embryo fibroblast proliferation was detected in the presence of liver microsomes from rabbit but not from mouse. The addition of a selective inhibitor of CYP1A1, α-naphthoflavone (4 μM), or furafylline (4 μM), to the incubation mixture abolished the thalidomide-induced suppression. Furthermore, addition of anti-rat CYP1A1 antibody also resulted in inhibition of suppression. The thalidomide-induced suppression was also observed with the microsomal system from human HepG2 cells pretreated with 3-methylcholanthrene (10 μM) but not from those pretreated with the vehicle. Both CYP1A1 and CYP1A2 proteins were detected in the rabbit liver microsomes by immunoblot analyses, but only CYP1A2 protein was detected in the mouse liver microsomes. In addition, CYP1A1 protein was detected in microsomes from HepG2 cells pretreated with 3-methylcholanthrene but not with the vehicle. These results strongly suggest the involvement of CYP1A1 in the thalidomide-induced suppression of embryo fibroblast proliferation.

Thalidomide is a potent human teratogen that led to limb malformation in newborns after maternal usage (McBride, 1961; Lenz, 1962). Thalidomide was removed from the market in 1961 when it was found to be a potent teratogen in humans. The biochemical mechanisms underlying its teratogenic effect are still unclear. Recently, there has been growing clinical interest in thalidomide due to its unique biological effects including anti-angiogenic, immunomodulatory and growth-suppressive effects (Schuler and Ehninger, 1995; Zwingenberger and Wnendt, 1996; Calabrese and Fleischer, 2000).

It is believed that metabolic activation of thalidomide is necessary for its biological effects, including teratogenesis and anti-angiogenesis. To understand the detailed mechanism for the bioactivation of thalidomide, extensive in vitro experiments were performed using several models including cytotoxicity of lymphocytes (Gordon et al., 1981), microvessel formation of aorta culture (Bauer et al., 1998), gap junctional intercellular communication of skin fibroblasts and epithelial cells (Nicolai et al., 1997; Onat et al., 2001), and attachment of tumor cells (Braun and Weinreb, 1984; Braun et al., 1986). Gordon et al. (1981) proposed that cytochrome P450-catalyzed oxidation of thalidomide to electrophilic arene oxide intermediate is involved in the teratogenicity of thalidomide. Lymphocyte toxicity of thalidomide was enhanced by the addition of inhibitors of microsomal epoxide hydrolase (mEH1) and prevented by adding purified mEH to the incubation mixture. Furthermore, Braun et al. reported that metabolites of thalidomide generated by P450 inhibited the cellular attachment that is an essential process for embryogenesis (Braun et al., 1986).

On the other hand, thalidomide teratogenicity in rabbits was reduced by the prostaglandin H synthase inhibitor, acetylsalicylic acid (Arlen and Wells, 1996; Wells et al., 1997) and free radical spin trapping agent, α-phenyl-N-r-butyl nitrotrone (Parman et al., 1999), suggesting the involvement of a prostaglandin H synthase-mediated free radical on thalidomide-induced teratogenicity. Furthermore, the thalidomide-induced anti-angiogenic effect in embryo bodies was suppressed by the coadministration of the hydroxy radical scavengers, mannitol and 2-mercaptoethanol, with thalidomide (Sauer et al., 2000).

These are species differences for thalidomide-induced teratogenicity. Rodents were resistant to the teratogenic effects of thalidomide, but rabbits, monkeys and humans were susceptible (Delahunt et al., 1965; Fratta et al., 1965; Schumacher et al., 1968). Parman et al. (1999) reported that thalidomide enhanced embryonic DNA oxidation in rabbits but not in mice even at a three times higher dose than that used in rabbits. Furthermore, thalidomide metabolites formed with human and rabbit microsomes were anti-angiogenic in aorta cultures, but metabolites formed by rat microsomes were not (Bauer et al., 1998).

Recently, we developed a system that we hope will be useful for detection of embryotoxins, by using primary cultures of embryo fibroblasts (Miyata et al., 2002b). Various embryotoxins, including benzo[a]pyrene and thalidomide, have trivial cytotoxicity in embryo

1 Abbreviations used are: mEH, microsomal epoxide hydrolase; SOD, superoxide dismutase; GD, gestational day; DMSO, dimethyl sulfoxide; P450, cytochrome P450; OD, optical density.
fibroblast systems, which at least in part is due to a lack of capacity for metabolic activation. Introduction of steps for microsomal preincubation and calcium precipitation (Cinti et al., 1972) prior to chemical contact resulted in a clear decrease in cell number by thalidomide and benzo(a)pyrene (Miyata et al., 2002b). Thalidomide causes oxidative DNA damage, which is likely to be an essential mechanism for thalidomide-induced teratogenesis (Parman et al., 1999). Oxidative DNA damage can lead to the cell cycle perturbation resulting in the suppression of embryonal cell proliferation (Little and Mirkes, 1992). Thus, we expect that the anti-proliferation assay in embryo fibroblasts may be able to serve as a model for teratogenesis of thalidomide.

In the present study, we used the embryo fibroblast anti-proliferation assay described above to investigate the role of enzymatic activation in thalidomide-induced suppression for embryo fibroblast proliferation. The present results suggest that a thalidomide metabolite(s) formed by CYP1A1 is involved in the suppression of embryo fibroblast proliferation.

Materials and Methods

Chemicals. Thalidomide was purchased from Tocris Cookson Inc. (Ballwin, MO). 1-Aminobenzotriazole, α-naphthoflavone, 2-melcaptoethanol, mannitol, 3,3′-diaminobenzidine, and neutral red were purchased from Sigma-Aldrich (St. Louis, MO). Fura-2 was purchased from Salford Ultrafine Chemicals and Research Ltd. (Manchester, UK). Dulbecco’s modified Eagle’s medium and penicillin-streptomycin were obtained from Invitrogen (Carlsbad, CA). 

Preparation of Microsomal Metabolites. Microsomes of liver and placenta were prepared as described previously (Miyata et al., 2002a). Rabbit at gestational day (GD) 19 and mice at GD14 were euthanized, the embryos placed in phosphate-buffered saline (pH 7.4), and the internal organs and heads were removed. The remaining torsos were minced and suspended in 0.25% trypsin for 40 min at 37°C. The reaction was stopped by the addition of an incubation medium (Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 100 U/ml penicilllin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, and 2 μM t-glutamine).

Preparation of Embryo Fibroblasts. Embryo fibroblasts were prepared as described previously (Miyata et al., 2002a). Rabbit at gestational day (GD) 19 and mice at GD14 were euthanized, the embryos placed in phosphate-buffered saline (pH 7.4), and the internal organs and heads were removed. The remaining torsos were minced and suspended in 0.25% trypsin for 40 min at 37°C. The reaction was stopped by the addition of an incubation medium (Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml amphotericin B, and 2 μM t-glutamine).

Inhibition Studies. Microsomal reaction mixture was preincubated with either one of P450 inhibitors (1-aminobenzotriazole, α-naphthoflavone, and furafylline) or with anti-rat CYP1A1 antibody, in the presence of substrate (thalidomide) for 5 min at room temperature prior to the initiation of the assay. 
P450 inhibitors dissolved in DMSO (1.2 μl) or the antibody diluted with phosphate buffer (12 μl) were added to the reaction mixture. Antioxidants (2-mercaptoethanol, mannitol, SOD, and catalase) dissolved in Dulbecco’s modified Eagle’s medium (12 μl) were added to the reaction mixture after microsomal incubation.

Measurement of Cell Number. The embryo fibroblasts were cultured at 37°C in an atmosphere of 5% carbon dioxide for 48 h. The cells were trypsinized and seeded into 96-well plates at a density of 8 × 103 cells/well in 100 μl of medium. Following incubation for 24 h, the culture medium was replaced with medium (100 μl/well) containing microsomal metabolites of thalidomide and incubated for an additional 48 h. The cells were treated with neutral red solution (50 μg/ml) for 3 h and then fixed with 1% formalin solution containing 1% CaCl2. After the neutral red was extracted with 50% ethanol containing 1% acetic acid, cell number of embryo fibroblasts was measured by absorbance at 540 nm (Borenfreund and Puerner, 1985). The absorbance of vehicle controls corresponding to 100% ranged between 0.33 optical density (OD) and 0.43 OD. All experiments were at least repeated twice.

Cell Proliferation Assay. Cell proliferation was determined by [methyl-3H]thymidine incorporation. The cells were seeded into 96-well plates and treated with microsomal metabolites as described for measurement of cell number. [methyl-3H]Thymidine (1 μCi/well) was added 48 h after the metabolite treatment. The cells were cultured for 2 h, and then 50 μl of 0.25% trypsin-EDTA was added. The plates were incubated for 10 min at 37°C before harvesting by use of a cell harvester. Incorporated radioactivity was counted in a Beckman LS 5000 scintillation counter (Beckman Coulter, Inc., Fullerton, CA). The incorporated radioactivity was normalized by cell numbers measured by neutral red assay.

Immunoblot Analysis. Microsomal proteins were prepared from mice at GD 14, rabbit at GD 19, and from HepG2 cells pretreated with 3-methylcholanthrene (10 μM), thalidomide (100 μM) or vehicle (DMSO) for 48 h. Microsomal proteins were subjected to electrophoresis on 8% SDS polyacrylamide gel and electrotransferred to nitrocellulose membranes. A polyclonal antibody against rat CYP1A1 was used to detect CYP1A1 and CYP1A2 proteins. After incubation with a horseradish-peroxidase-conjugated goat anti-rabbit IgG (American Qualex, San Clemente, CA), the immuno-reactive bands were detected by 3,3′-diaminobenzidine.

Statistical Analysis. Results are expressed as the mean ± S.D. for each culture well (n = 12 or 7) and analyzed by the unpaired Student’s t test. A P value of less than 0.05 was considered as a limit for statistical significance in this study.

Results

Effect of Thalidomide Metabolites on Cell Number of Rabbit Embryo Fibroblasts. Previous in vitro studies have shown that bioactivation is necessary to exert several biological effects of thalidomide, including teratogenesis and anti-angiogenesis. To determine whether thalidomide is metabolically activated by microsomal drug-metabolizing enzymes in rabbit placenta, or rabbit embryo or maternal liver, rabbit embryo fibroblasts were exposed to thalidomide metabolites produced in the presence of those microsomes. A significant decrease in embryo fibroblast cell number was detected after contact with supernatants of maternal liver microsomal incubation mixtures but not those of embryo liver or placenta microsomal incubation mixtures (Fig. 1).

Cell Growth of Mouse Embryo Fibroblasts. Cell growth of mouse embryo fibroblasts relative to the time of treatment with supernatants of rabbit liver microsomes preincubated with thalidomide was analyzed (Fig. 2). Supernatants of microsomes incubated without thalidomide stimulated mouse embryo fibroblast proliferation compared with normal medium. Although the addition of NADPH-generating system in the incubation mixture cause a slight decrease in the proliferation of embryo fibroblasts, it is seen that the embryo fibroblasts proliferate vigorously for 48 h after treatment with the...
incubation mixture (control). Significant decreases in embryo fibroblast cell numbers were found 24- and 48-h after contact with supernatants of rabbit liver microsomes incubated with thalidomide (100 μM).

The reactive intermediates of thalidomide are likely to be unstable, because a clear decrease in the suppressive effect was observed in delayed contact between embryo fibroblast and preincubated mixture. The suppressive effect was reduced by approximately 50% when embryo fibroblasts were treated with the preincubated mixture at 15 min after the addition of CaCl₂. The effect disappeared by placement of the preincubated mixture at 25°C for 1 h. Thus, all contacts between embryo fibroblast and preincubated mixture were performed within 5 min after the addition of CaCl₂.

**Materials and Methods.**

Cell growth was measured by the method described under Materials and Methods. Rabbit embryo fibroblasts were treated with supernatants of the reaction mixture for 48 h. Cell number of embryo fibroblasts was measured by absorbance at 540 nm. Data are expressed as a percentage of control. The control indicates cell number of embryo fibroblasts treated with supernatants of each microsome incubated with vehicle (DMSO). Values represent the mean ± S.D. of 12 culture wells. Significant difference from vehicle control (**, p < 0.01).

Species Differences in Effects of Thalidomide Metabolites. Rabbits and humans are known to be susceptible to the teratogenic effect of thalidomide, while rodents were resistant (Delahunt et al., 1965; Fratta et al., 1965; Schumacher et al., 1968). No significant decrease was found in cell numbers of mouse embryo fibroblasts after contact with supernatants of mouse liver microsomes preincubated with 30 or 100 μM thalidomide (Fig. 3). In similar experiments, a 50% decrease in cell number was found in the embryo fibroblasts exposed to supernatants of microsomal reaction mixtures containing 100 μM benz[a]pyrene (a positive control). When mouse embryo fibroblasts were treated with supernatants of rabbit, instead of mouse, maternal liver microsomal reaction mixtures, a clear decrease in cell number was detected as shown in Fig. 3. These results suggest that species differences in susceptibility to thalidomide proliferative responses are likely to derive from biotransformation of thalidomide rather than embryo fibroblast susceptibility.

**Cell Proliferation Inhibition by Thalidomide Metabolites.** To define the cause of the decrease of embryo fibroblast cell number, cell proliferation was analyzed by [³H]thymidine incorporation, [³H]Thymidine incorporation normalized in cell numbers of embryo fibroblasts was significantly reduced by treatment with the rabbit liver metabolites derived from more than 30 μM thalidomide (Fig. 4). Furthermore, the decrease was dose-dependent. These results confirm the anti-proliferative effect of the thalidomide metabolites.

**Involvement of CYP1A Forms.** Previous in vitro studies suggested the involvement of P450s in the bioactivation of thalidomide. Thus, the role of P450s in the formation of thalidomide metabolites, which suppressed cell proliferation, was examined using P450 inhibitors or anti-P450 antibody. The deletion of the NADPH-generating system in the preincubation mixture abolished the suppressive effect of thalidomide on the proliferation of embryo fibroblasts (data not shown). Furthermore, the addition of P450 inhibitor, 1-aminobenzo-
Thalidomide concentration in incubation mixture was indicated. The embryo fibroblasts were cultured with [3H]thymidine for 2 h following treatment with thalidomide metabolites for 48 h. [3H]Thymidine incorporation was normalized to control, representing the mean ± S.D. of 12 culture wells. Significant difference from vehicle control (**, p < 0.01).

Expression of CYP1A Forms. To determine microsomal CYP1A level, immunoblot analyses were carried out using anti-rat CYP1A1 antibody. Immunoreactive bands to anti-rat CYP1A1 antibody were detected in microsomal proteins from the liver of mouse and rabbit but not from rabbit placenta and embryo (Fig. 8). Two immuno-reactive proteins corresponding to CYP1A1 and CYP1A2 were detected in rabbit liver and in β-naphthoflavone-treated mouse liver, but only a lower band was detected in liver microsomes of untreated mice. These results are consistent with previous reports showing constitutive expression of CYP1A2 but not CYP1A1 in mouse livers (Dey et al., 1999) and of both CYP1A1 and CYP1A2 in rabbit livers (Rey-Grobellet et al., 1996). Thus, the lower band was assigned as CYP1A2. On the other hand, a single band identical to electrophoretic mobility of human CYP1A1, but not CYP1A2, was detected in microsomes from HepG2 cells treated with 10 μM 3-methylcholanthrene but not those from HepG2 cells treated with 100 μM of thalidomide or vehicle.

Involvement of Microsomal Epoxide Hydrolase. mEH is expressed in embryo fibroblasts (Miyata et al., 2002a). The toxicity of thalidomide toward lymphocytes was enhanced by the addition of inhibitors of mEH (Gordon et al., 1981). Thus, embryo fibroblasts from mEH-null mice were treated with supernatants of rabbit liver microsomes preincubated with thalidomide to verify the involvement of mEH in the detoxification of thalidomide-reactive intermediates. No significant difference in cell numbers of embryo fibroblasts treated with the rabbit liver microsomal reaction mixture was observed between mEH-null and the wild-type mice (data not shown). Furthermore, the addition of mEH inhibitor, 1,2-epoxy-3,3,3-trichloropropane, to the preincubation mixture did not alter the thalidomide-induced suppressive effect.

Involvement of Radical Species. If reactive oxygen species and/or free radicals are involved in the thalidomide-induced suppression of cell proliferation, the effect of thalidomide could be diminished by the addition of a hydroxyl radical and/or free radical scavengers to the incubation mixture. As described in Table 1, thalidomide-induced suppression of cell proliferation was almost completely abolished by the addition of hydroxyl radical scavenger, 2-mercaptoethanol (50 μM), to the reaction mixture after preincubation. SOD and catalase, or mannitol, also partially abolished thalidomide-induced suppression of cell proliferation.

Discussion

To understand the role of bioactivation of thalidomide, the suppression of embryo fibroblast proliferation was analyzed using a newly developed preincubation system (Miyata et al., 2002b). We used primary cultured embryo fibroblasts instead of homogeneous cell line

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such as C3H10T1/2 derived from murine embryo. Thalidomide preincubated with rabbit liver microsomes caused more than a 20% decrease in mouse or rabbit embryo fibroblast proliferation. Although the suppressive effects of thalidomide were modest, thalidomide caused a dose-dependent suppression of embryo fibroblast proliferation. Because embryo fibroblasts consist of heterogeneous-differentiated populations, only a portion of the embryo fibroblasts might respond to the incubation mixture of thalidomide.

The suppressive effect of thalidomide metabolites depended on the sources of animal species of liver microsomes for preincubation of thalidomide but not on the species of embryo fibroblasts. These results provide convincing evidence that microsomal drug-metabolizing enzymes are critical for species difference in the thalidomide-induced suppression. Furthermore, the addition of CYP1A inhibitors (furafylline and α-naphthoflavone) and anti-rat CYP1A1 antibody reversed the suppression, suggesting the involvement of CYP1A forms in the production of reactive thalidomide metabolites. CYP1A1 and CYP1A2 were constitutively expressed in rabbit liver (Rey-Grobellet et al., 1996). In rabbit liver microsomes, CYP1A1 and CYP1A2 proteins were detected by anti-rat CYP1A1 antibody, which inhibited the suppressive effect of thalidomide (Fig. 8). These results support the idea that rabbit CYP1A1 and/or CYP1A2 were involved in the bioactivation of thalidomide related to suppression of cell proliferation. Furthermore, the thalidomide-induced suppression was observed in the preincubation system using microsomes prepared from 3-methylcholanthrene-treated HepG2 cells, which expressed CYP1A1 protein but not CYP1A2 protein. CYP1A2 protein is not detected in HepG2 cells even after polyaromatic hydrocarbon induction (Va-
significant effect of thalidomide on cytotoxicity and proliferation were evaluated using mouse fibroblastoid L929 cells resulting in no significant effect of thalidomide on cytotoxicity and proliferation (Walmdorf et al., 2002). These results are consistent with our experimental data on the direct treatment of embryo fibroblast with thalidomide (Miyata et al., 2002b). We do not know whether the thalidomide metabolites detected in the embryo fibroblast assay system can also cause thalidomide-induced teratogenesis and/or anti-angiogenesis. However, the present results in the system of embryo fibroblasts were consistent with reported species differences in thalidomide-induced teratogenesis (Delahunt et al., 1965; Fratta et al., 1965; Schumacher et al., 1968) and anti-angiogenesis (Bauer et al., 1998).

In this study, we present evidence that CYP1A1-mediated metabolism is involved in the thalidomide-induced suppression of embryo fibroblast proliferation. We are currently identifying the structure of CYP1A1-mediated thalidomide metabolites causing the suppression.

Acknowledgments. We thank Dr. Frank J. Gonzalez of National Institutes of Health (Bethesda, MD) for kindly supplying mEH-null mice.

References


Lewinsky E and Milstein P (1981) Effect of 4-hydroperoxyxylcophosphohemate (4-OH-CP) and 4-hydroperoxylxylcophosphohemate (4-OH-DCP) on the cycle of postimplanta


TABLE 1

Effect of antioxidants on thalidomide-induced cytotoxicity to mouse embryo fibroblasts

<table>
<thead>
<tr>
<th>Additions</th>
<th>Cell Viability (A590 nm)</th>
<th>Percentage of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMSO (control)</td>
<td>Thalidomide (100 µM)</td>
</tr>
<tr>
<td>None</td>
<td>0.41 ± 0.04</td>
<td>0.31 ± 0.03</td>
</tr>
<tr>
<td>2-Mercaptoethanol (50 µM)</td>
<td>0.40 ± 0.04</td>
<td>0.41 ± 0.03</td>
</tr>
<tr>
<td>Mannotol (5 mM)</td>
<td>0.42 ± 0.02</td>
<td>0.38 ± 0.04</td>
</tr>
<tr>
<td>SOD (200 U/mg) + Catalase (500 U/mg)</td>
<td>0.39 ± 0.03</td>
<td>0.34 ± 0.02</td>
</tr>
</tbody>
</table>

* Significant differences (p < 0.01) from percentage of control of none (no antioxidants).