Effect of 2-Methoxyestradiol (2ME) an anti-angiogenic agent on in vivo tumour bearing mouse

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2-Methoxyestradiol (2-ME), once considered an inactive end metabolite of estradiol, has emerged as a very promising agent for cancer treatment. 2 ME was reported to inhibit the proliferation of cells in different tumour cell lines. But it is necessary to investigate the effect of 2ME on in vivo system. So the present study was undertaken to evaluate the efficacy and safety of 2ME on in vivo mouse tumour model considering tumour growth rate with nature of vascular density, mouse survival rate and bone marrow toxicity. Tumour regression in response to different concentrations of 2ME was studied at different time intervals by morphometric analysis of tumor size. A steady decrease in tumour growth was noted after the treatment of 0.1mg ME in tumour bearing mouse which was correlated with the gradual increase of mouse survival or life span. In addition, 0.1mg ME not only induced a strong anti-angiogenic response by a decline in the number of blood vessels in the tumour but also protect bone marrow by inhibition of maximum numbers of affected cell with chromosomal aberrations. So 2-Methoxyestradiol may be applied as a novel therapeutic drug for cancer.

Key words: 2-Methoxyestradiol, angiogenesis, morphometric analysis, mouse tumour model, chromosomal aberrations.

INTRODUCTION

The process of angiogenesis (i.e. the formation of new blood vessels) plays a critical role in both normal physiological events as well as in many pathological processes such as tumour growth and metastasis (Folkman, 1989). The objective of the anti-angiogenic therapy is quite different in comparison to other therapeutic approaches as anti-angiogenic drugs stop new blood vessels from forming around the tumour and break up the existing network of the abnormal capillaries which deliver nutrients and oxygen to the rapidly growing tumour cells. (Ferrara, 1995; Folkman,1995). Different types of anti-angiogenic compounds effective in animal studies have been developed, in many cases, however, the basis of their activity is uncertain.

2-Methoxyestradiol (2-ME), a metabolite of 17 β-estradiol (E₂), once considered an inactive end metabolite, has recently emerged as a very promising agent for cancer treatment (Bruce et al., 2012). 2-ME targets rapidly growing cells with relatively high specificity and does not kill non-dividing cells. The effect of 2ME on the immune system has been investigated in animal models (Duncan et al., 2012; Stubelius et al., 2012; 2014). In these models, 2ME inhibited local inflammation, angiogenesis and leukocyte infiltration etc. The effect of different concentrations of 2ME at various time points on some tumour cell lines (viz. MCF-7 and GH3) has been studied earlier (Banerjee et al., 2002; 2003; Banerjee and Banerjee, 2005; 2008). Low concentration of 2ME (1µM) significantly increased the vascular endothelial growth factor (VEGF) m-RNA and protein expression whereas decreasing expression of VEGF m-RNA and protein expression was noted with higher (5-10 µM) 2ME concentrations. But it is necessary to investigate the effect of 2ME on in vivo system. Till now little has been done on the effect of 2ME on somatic chromosome of the tumor bearing host, particularly during the period of tumor regression (Banerjee et al., 2006). Therefore, the present
Table 1. Treatment schedule (treatment was started at log-phase of tumour growth, i.e. after 8th day of tumour cell injection; treatment continued for 5 consecutive days).

<table>
<thead>
<tr>
<th>Nontreated (NT) or Control / Drug</th>
<th>Route</th>
<th>Dose</th>
<th>Average weight of Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT or Control (solution prepared with absolute alcohol and distilled water v/v 1:1 ratio)</td>
<td>IP</td>
<td>0.2ml/mouse</td>
<td>15 gms</td>
</tr>
<tr>
<td>2-Methoxyestradiol (2-ME) low dose</td>
<td>IP</td>
<td>0.05mg ME/Mouse</td>
<td>15 gms</td>
</tr>
<tr>
<td>2-Methoxyestradiol (2-ME) Medium dose</td>
<td>IP</td>
<td>0.1mg ME/Mouse</td>
<td>15 gms</td>
</tr>
<tr>
<td>2-Methoxyestradiol (2-ME) high dose</td>
<td>IP</td>
<td>0.5mg ME/Mouse</td>
<td>15 gms</td>
</tr>
</tbody>
</table>

study was aimed to investigate the dose dependent effect of 2 ME on in vivo S-180 cell line injected solid mouse tumour model considering mouse survival, tumour growth rate and bone marrow toxicity as end point. Additionally, the efficacy and safety of 2-ME was determined by studying the data whether 2ME can give significant protection against mutagenicity and carcinogenicity.

MATERIALS AND METHODS

Experimental animal

Inbred adult mice (Mus musculus) of both sexes, 4-6 weeks of age were used for experiment. Mice were maintained under controlled conditions of temperature and were provided with standard mice feed and water ad libitum. Before experimental work the mice were kept for 10 days under normal healthy laboratory conditions for acclimatization.

Selection of Animal tumour

A suitable mouse (Mus musculus) tumour model (Sarcoma 180 ascities cell line) was selected for experiment. Sarcoma-180 cell lines were obtained from Chittaranjan National Cancer Institute, Kolkata, India and maintained in the laboratory by serial passages.

Induction of solid tumour in mouse

The ascites form of sarcoma 180 (S-180) cells (inoculum 1×10⁶ cells per animal) was injected subcutaneously in leg ventrally for tumour induction. The whole process was carried out aseptically. The mean time for appearance of palpable leg tumour was 7-8 days as standardized in the laboratory (Banerjee and Mallick, 2013). Twenty four (24) mice (both male and female equally) of same age group were taken for the present experiment. Six (6) specimens were taken for each set of treatment. All experiments were performed in accordance with the guidelines formulated by the Institutional animal Ethics Committee for the care and use of Laboratory animals.

Drug or Chemical

2-Methoxyestradiol (Sigma, U.S.A), an anti-angiogenic end metabolite of estradiol was chosen. It is synthesized by sequential hydroxylation of the parent compounds followed by methylation in liver (Brueggemeier and Singh, 1989).

Treatment of 2ME and experimental protocol

Treatment was started at log-phase of tumour growth, i.e. after 8th day of tumour cell injection. Solid leg tumour bearing mice was subjected to intraperitoneal injection of 2ME stock solution (three doses i.e. low, medium and high) once daily for five consecutive days.

Preparation of ME solution

5mg ME was dissolved in 5ml absolute alcohol. Then the solution was diluted in 5ml sterilized distilled water and ME stock solution was prepared. A parallel control (solution prepared with absolute alcohol and distilled water v/v 1:1 ratio) was made to analyze the effect of the solvent (Table 1).

Experimental animals (24 mice) were divided into four groups:

Group 1 (Control): Tumour bearing mice were not given any drug. Normal feeding was provided.

Group 2 (Low ME dose): 0.05 mg ME was given through solution intraperitoneally to each tumour bearing mouse once daily for five consecutive days. Mice were fed with normal food.

Group 3 (Medium ME dose): 0.1 mg ME was given through solution intraperitoneally to each tumour bearing mouse once daily for five consecutive days. Mice were fed with normal food.

Group 4 (High ME dose): 0.5 mg ME was given through solution intraperitoneally to each tumour bearing mouse once daily for two to three consecutive days. Mice were fed with normal food.

Mouse survival

Mouse survival or the life span of tumour bearing mouse in both non-treated or control and treated groups was studied according to the specific protocol (Basu et al., 2004; Chakroborty et al., 2004).

Tumour regression

Tumour regression in response to therapy in both non-
treated or control and treated groups was studied at different time intervals by morphometric analysis of tumour size.

**Tumour volume measurement**

The tumour growth or volume was measured in cubic cm using the formula: tumour volume = width × length × 0.5 width (Ray et al., 2005). The volume was measured once daily for five consecutive days using Vernier calipers. Depending on the experiments, 5 to 6 mice per group were used.

**Bone marrow toxicity assessment**

Bone marrow toxicity was measured by chromosomal aberration analysis (Banerjee et al., 2006; Banerjee and Banerjee, 2008). Actually one of the pathways for the action of most antineoplastic agents is chromosomal aberration. So observations on the effect of antineoplastic agents on the chromosome of host own body cells will provide valuable information for better monitoring of cancer therapy. Chromosome preparation from bone marrow cells of tumour bearing mice in both control and treated series was done according to the method as practised by earlier workers (Chakrabarti et al., 1985; Chakrabarti and Chakrabarti, 1987; Banerjee and Chakrabarti, 2004). In brief, all specimens received i.p. injection of 0.04 % Colchicine (Sigma, St Louis, U.S.A) solution at a rate of 1 ml /100 gm body weight 1.5 hr prior to sacrifice. Cells from bone marrow were collected in hypotonic solution (0.075M KCl) and aspirated gently to form a homogenous cell suspension. Then cell suspension was incubated for 30 min. at 37°C and followed by centrifugation at 1500 rpm for 15 min. The pellet was fixed in fresh methanol: acetic acid fixative (3:1 v/v). The whole process was repeated thrice. Three drops of cell suspension were dropped on clean grease-free slide (soaked previously in chilled 50% ethanol) and allowed to dry in flame. For conventional staining slides were dipped into 5% phosphate buffered Giemsa stain (pH 6.8) for 40 min. and washed in tap water for observation under the Binocular Research Microscope (10 × 100 magnification).

Criteria for selecting metaphases for scoring of data: Only those metaphases displaying well differentiated dark stained sister chromatids and in which chromosomes showed good spread with little or no overlapping were chosen for study.

**Study of tumour vasculature**

Tumour tissues of both non-treated and treated groups were excised with sharp blade and kept in 0.9% normal saline for 1 minute. Then excised tumour tissues were observed under the Binocular Research Microscope (5 × 10 magnification) to study the nature of tumour vessels according to the method as practiced in the laboratory.

**Statistical analysis**

All data are expressed as mean and standard error (SE). Statistical analyses of survival test and morphometric analysis of tumour growth are made by Student’s t-test (Panse and Sukhatme, 1985). Significance is indicated by an asterisk.

**RESULTS**

**Solid tumour induction in mouse**

The mean time for appearance of palpable leg tumour was 7-8 days.

**Effect of 2ME on Mouse survival**

Treatment with 0.1mg ME/mouse showed considerable increase of life span of tumour bearing mouse when compared with non-treated and other treated group. The survival time was 17.2±0.58 (days, mean ± standard error, N=5) in the 0.1mg ME treated group (Figure 1). The survival time of 0.1mg ME treated series was significantly longer than control and other treated groups (P<0.001).

**Tumour growth regression**

Tumour growth was analysed on and from 8 th days to 12 th days after tumour cell injection. The non-treated group showed increase of tumour growth (3.5 ± 0.87) where as 0.1mg ME treatment showed regression in tumour volume (1.10 ± 0.23). A t-test showed that this difference between non-treated group and 0.1 mg ME treatment group was significant (P<0.05). No changes in tumour volume were observed in both 0.05 mg ME and 0.5 mg ME treated groups. Interestingly, 0.5 mg ME induced toxic effect as most of the specimens were expired on 8 th and 9 th day (Figure 2).

**Chromosomal aberration study to assess bone marrow toxicity**

Bone marrow toxicity test was done through the scoring of different types of simple chromosomal aberration (SCA) and complex chromosomal aberration (CCA) to monitor the action of drug (Table 2). Chromatid breaks, lesions, deletions are included within the SCA type where as centric fusion or formation of metacentric and submetacentric chromosomes, exchanges, pulverizations are included within the CCA type. It was interesting to note that 0.5mg ME treated tumour bearing mouse revealed a large numbers of affected metaphase cells (Figure 3a,b,d,f) with maximum number of simple and complex chromosomal aberrations when compared with 0.1mg ME treated mouse (Figure 3a,b,e).
Figure 1: The survival time (days) of non-treated or control and treated tumour bearing mouse. NT = non-treated; ME = methoxyestradiol: 0.05mg ME (low dose); 0.1mg ME (medium dose) and 0.5mgME (high dose). The survival time was 17.2±0.58 (days, mean ± standard error, N=5) in the 0.1mgME treated group. The survival time of 0.1mg ME treated series was significantly longer than NT or control and other treated groups (**P<0.001).

Figure 2: Morphometric analysis of tumour growth in non-treated or control and treated tumour bearing mouse. NT = non-treated; ME = methoxyestradiol: 0.05mg ME (low dose); 0.1mg ME (medium dose) and 0.5mgME (high dose). Tumour growth was analysed on and from 8th days to 12th days after tumour cell injection. The NT group showed increase of tumour growth where as 0.1mg ME treatment showed regression in tumour volume significantly (*P<0.05). No changes in tumour volume were observed in both 0.05 mg ME and 0.5 mg ME treated groups. In addition, 0.5 mg ME induced toxic effect as most the specimens were expired on 8th and 9th day.

Table 2. Number of normal metaphase cells (N.C.), affected metaphase cells (A.C.), simple chromosomal aberrations (SCA) and complex chromosomal aberrations (CCA) in NT or control and different treated tumour bearing mouse. Each entry represents the mean ± SE.

<table>
<thead>
<tr>
<th>Doses</th>
<th>N.C</th>
<th>A.C</th>
<th>SCA</th>
<th>CCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>38 ± 0.58</td>
<td>12 ± 0.58</td>
<td>8.67 ± 0.34</td>
<td>2.67 ± 0.34</td>
</tr>
<tr>
<td>0.05 mg ME</td>
<td>37.67 ± 0.34</td>
<td>12.33 ± 0.34</td>
<td>7.33 ± 0.34</td>
<td>2.33 ± 0.34</td>
</tr>
<tr>
<td>0.1 mg ME</td>
<td>39.33 ± 0.34</td>
<td>10.67 ± 0.34</td>
<td>7.67 ± 0.34</td>
<td>2.67 ± 0.34</td>
</tr>
<tr>
<td>0.5 mg ME</td>
<td>33.67 ± 0.34</td>
<td>16.33 ± 0.34</td>
<td>10.67 ± 0.34</td>
<td>5 ± 0.58</td>
</tr>
</tbody>
</table>
Study of tumour vasculature to analyse the anti-angiogenic effect of 2-Methoxyestradiol

The tumour sections of control or non-treated tumour bearing mouse showed maximum number of blood vessels where as tumour sections of 0.1 mg ME treated tumour bearing mouse revealed fewer blood vessels (Figure 4a, b, c). So 0.1 mg ME treatment induced a strong antiangiogenic response in such tumour bearing mouse by a significant decrease in tumour vasculature density.
DISCUSSION

Anti-angiogenic drugs stop new blood vessels growth around a tumour and break up the existing network of abnormal capillaries that feeds the tumour. The first clinical trial of an anti-angiogenic drug – interferon alpha began for the treatment of hemangioma in infants (Ricketts et al. 1994). In 1996 another drug TNP-470 - synthetic analogue of the substance fumagillin showed inhibitory effect of endothelial cell proliferation and angiogenesis (Stepien et al., 1996). 2-Methoxyestradiol has been tested not only in some animal models of different diseases i.e. rheumatoid arthritis, multiple sclerosis etc. (Brahn et al., 2008; Stubelius et al., 2011; Duncan et al. 2012) but also in some tumour cell lines i.e. MCF-7 and GH3 (Banerjee et al., 2002; 2003) cell lines.

In our study we have used three doses of ME: i) low dose 0.05mg ME, ii) medium dose 0.1mg ME and iii) high dose 0.5mg ME to investigate its efficacy. The results indicate that medium dose (0.1mg ME concentration) is highly effective in inhibiting blood vessel growth, as shown in Figure 3f.
potent antineoplastic and antiangiogenic drug. The regression of the tumour during the course of therapy was determined which is correlated with the gradual increase of mouse survival or life span of 0.1mg ME treated tumour bearing mouse. Daily 2ME treatment (0.1mg ME for 5 consecutive days) induced a strong anti-angiogenic response as shown by a decrease in tumour vascular density or by a decline in the number of blood vessels in tumour. Analysis of bone marrow metaphases clearly points out that 0.1 mg ME can protect bone marrow as it inhibits different types of chromosomal aberrations.

No significant changes in the survival rate of mouse or life span of tumour bearing mouse and tumour volume were observed in NT or control, 0.05mg and 0.5mg ME treated groups. Interestingly, 0.5mg dose has exerted toxic effect as most of the tumour bearing mice were expired within 2nd or 3rd day of the treatment.

So, the present result is in agreement with the previous findings as it was studied on some in vitro tumour cell lines i.e. MCF-7 and GH3 (Banerjee et al., 2002; 2003). Overall, we conclude that 0.1mg ME is more effective than 0.05mg and 0.5 mg ME not only in controlling the tumour regression with blood vessels proliferation but also in prolonging survival time or life span of tumour bearing mouse. Moreover, 0.1mg ME treated bone marrow cells revealed a significantly less number of affected metaphase cells with minimum number of chromosomal aberrations when compared with 0.5mg ME treated mouse. So 2ME may be applied as a novel therapeutic drug for cancer.

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