Influence of Prostaglandin A2 and 2-Methoxyestradiol on Telomerase Activity in Cancer Cell Lines and a Non-tumorigenic Epithelial Breast Cell Line

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ABSTRACT
Telomerase activity plays an important role in the regulation of cell senescence by limiting the number of divisions. Significant levels of telomerase activity have been detected in 85% to 90% of human cancers, however none or negligible amounts of telomerase activity were observed in benign tumors and healthy tissue. This study investigated the levels of telomerase activity in three cancer cell lines and a non-tumorigenic cell line after exposure to two endogenous metabolites respectively, namely 2-methoxyestradiol (ZME) and prostaglandin A2 (PGA2). Cells were exposed to either 1 μM ZME or 20 μg/ml PGA2 and their relative telomerase activity (RTA) was measured by means of a combined method including the polymerase chain reaction and an enzyme-linked immunosorbent assay. PGA2 exposure caused a statistically significant decrease in the RTA of the three cancer cell lines (HeLa, WHCO3, MCF-7) (P < 0.05). In contrast, the RTA of the non-tumorigenic MCF-12A cell line was statistically significantly increased after exposure to PGA2 (P < 0.00005). The RTA of HeLa, WHCO3 and MCF-12A cells were significantly decreased after ZME exposure (P < 0.05). These results confirm that PGA2 and ZME could be of potential value as anti-tumor agents, since both exert an inhibitory activity on the tumor-associated function of the telomerase enzyme involved in cancer cell immortality.

The telomerase enzyme plays an important role in the assembly of tandem repeats (TTAGGG)n on chromosome ends called telomeres (1). These telomeric repeat sequences are responsible for the integrity of the genome by preventing nucleolytic degradation, ligation or chromosome fusion (22). Telomere length decreases by approximately 50–200 nucleotides per cell division in differentiated somatic cells. These repeat DNA sequences can therefore be regarded as signals for an irreversible growth arrest termed cellular senescence. Cell death is induced when a critical length is reached (1).

The telomerase enzyme is a ribonucleoprotein DNA polymerase that elongates telomeres in eukaryotic cells (27). The enzyme consists of two components namely the human telomerase RNA and a catalytic subunit, human telomerase reverse transcriptase. Significant levels of telomerase activity have been observed in germ line cells that give rise to eggs and sperm, as well as in immortalised cells. Analyses of cancer cell lines and excised tumors have shown increased levels of telomerase activity, linking telomerase activation to the transition from normal to transformed and to the immortal cancer phenotype (19, 33). An increase in mRNA expression of the telomerase reverse transcriptase catalytic subunit, as well as enhanced levels of telomerase activity were observed in the majority of cervical carcinomas and in a subset of high-grade premalignant cervical lesions (31, 34). Escape from cellular senescence and immortalisation of cells via telomerase ac-
tivation can possibly be regarded as additional steps in oncogenesis that most tumors require for continuous proliferation. Telomerase is thus not only a target for the diagnosis of cancer, but also for the development of novel therapeutic anti-cancer agents (3).

In this study, the effects of two endogenous metabolites, namely prostaglandin A2 (PGA2) and 2'-methoxyestradiol (2ME) were investigated on telomerase activity of three cancer cell lines, as well as a spontaneously immortalised non-tumorigenic cell line. 2ME, an endogenous metabolite of 17 beta-estradiol, is an anti-mitotic drug and tubulin poison that inhibits growth and induces apoptosis in a large variety of tumor and non-tumor cell lines in vitro (18, 20, 21, 29, 30). These effects are observed in estrogen receptor (ER) positive, as well as ER negative cell types. Furthermore, 2ME has been shown to be an effective oral anti-angiogenic and anti-tumor agent in vivo models (5, 7, 13). 2ME is the only estrogen metabolite devoid of uterotrophic, estrogenic and tumorigenic activity in vivo (3). Toxic effects (e.g. hair loss, gastrointestinal disturbance, inhibition of leucopoiesis) were not experienced in any of the experiments (5, 7, 13). Recently, it has been shown that 2ME is responsible for the upregulation of death receptor 5 expression in vitro and in vivo, thus activating the extrinsic pathway of apoptosis. 2ME is currently included in Phase I clinical trials under the name PANZEM™ (11).

PGA2, another endogenous metabolite derived from arachidonic acid, was also shown to exhibit potent anti-proliferative activity in vitro (9, 17, 28) and in vivo (2, 23, 24, 26). PGA2 inhibited proliferation of tumor cells depending on close, duration of exposure and cell type (15). We have shown that breast adenocarcinoma cells (MCF-7) were more susceptible to the anti-mitogenic effects of PGA2 when compared to human epithelial cervix carcinoma (HeLa) cells and suggested two possible pathways of apoptosis induction, namely enhanced activation of CDK 2-cyclin A in S-phase-arrested cells and ceramide-linked apoptosis (G2/M-phase) (14). Degree of differentiation of oesophageal carcinoma cells was shown to influence the susceptibility of tumor cells to the anti-mitogenic effects of PGA2. More pronounced effects were observed in less differentiated cell lines, while more differentiated and normal cells appeared to be less affected (15).

Both endogenous metabolites target active proliferating cells and play an active role in the induction of apoptosis, especially in cells that present with carcinogenic properties (15, 30, 35). The characteristics of PGA2 and 2ME render them as possible anti-tumor agents when compared to conventional chemotherapeutic treatments. Since telomerase activation is linked to the transition from normal to the immortal cancer phenotype, the effects of these endogenous metabolites on telomerase activity were therefore studied in three cancer cell lines as well as a non-tumorigenic epithelial breast cell line.

MATERIALS AND METHODS

Cell lines. HeLa (human epithelial cervix carcinoma) was purchased through Sterilab Services (Johannesburg, SA.) from the American Tissue Culture Collection (ATCC) (Maryland, USA). MCF-7 cells were derived from a pleural effusion of human breast adenocarcinoma and supplied by Highveld Biological (Sandringham, SA). The WHCO3 cell line (a poorly differentiated non-keratinising squamous oesophageal cell carcinoma) was a gift from Professors Thomley and Veale (Department of Zoology, University of the Witwatersrand, Johannesburg, SA). WHCO3 cells were obtained through a biopsy from a patient with squamous oesophageal carcinoma. The MCF-12A cell line is a non-tumorigenic epithelial cell line produced by long-term culture of normal mammary tissue. This cell line was a gift from Professor Parker (Division of Medical Biochemistry, University of Cape Town, Cape Town, SA).

Cell culture maintenance and chemicals. PGA2, 2ME, minimum essential medium eagle (MEM) with Earle's salts, L-glutamine and NaHCO3, Ham's F12 medium, Trypsin-EDTA, epidermal growth factor, cholera toxin, insulin, hydrocortisone and Trypan blue were supplied by Sigma Chemical Co. (St. Louis, USA). Heat-inactivated fetal calf serum, sterile cell culture flasks and plates were obtained through Sterilab Services (Johannesburg, SA). Phosphate buffered saline (PBS) was purchased from Gibco BRL through Laboratory Specialist Services (Johannesburg, SA). Dulbecco's modified Eagle's medium, penicillin, streptomycin and fungizone were obtained from Highveld Biological (Sandringham, SA). All other chemicals were of analytical grade and supplied by Sigma Chemical Co. (St. Louis, USA).

Telomerase assay. The TeloTAGGG Telomerase PCR ELISAPLUS kit was supplied by Roche (Mannheim, Germany).
Culturing of cell lines. Cells were grown as monolayers in minimum essential medium, at 37°C in a humidified atmosphere containing 5% CO₂. MEM was used for the three cancer cell lines and supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 μg/ml), streptomycin (100 μg/ml) and fungizone (250 μg/ml).

MCF-12A media consisted of a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 10 μg/ml insulin and 500 ng/ml hydrocortisone. Stock solutions of PGA₂ and 2ME were prepared in ethanol and dimethyl sulfoxide (DMSO) respectively. The solvent concentrations in the media never exceeded 0.05%. In all the experiments concentrations of 20 μg/ml PGA₂ and 1 μM 2ME were used, since dose-dependent studies (unpublished data) showed maximum growth response at these dosages in vitro.

PGA₂ - and 2ME exposure of cells. Known numbers of viable cells were seeded in appropriate culture vessels and incubated for 24 h. Cells were harvested after 16 h of exposure to vehicle controls, 1 μM 2ME or 20 μg/ml PGA₂. Concentration-dependent studies from previous research showed that these concentrations result in optimal growth inhibition in vitro (6, 14). A sample of cells were stained with Trypan blue and counted using a hemacytometer. The remaining cells were centrifuged at 3000 × g for 5 minutes at 4°C and the supernatant was removed. Cells were resuspended in PBS and centrifuged as described above before measurement of telomerase activity.

Measurement of telomerase activity. Telomerase activity was measured in cell lines described above using the Telomerase PCR ELISA kit according to the manufacturer's instructions. Elongation products generated through the addition of telomeric repeats (TTAGGG) by telomerase were amplified using the Telomeric Repeat Amplification Protocol (TRAP) in a GeneAmp® PCR System 9700 PE Applied Biosystems (Norwalk CT, USA). Polymerase chain reaction (PCR) products were detected utilising a photometric enzyme immunoassay for semi-quantitative determination of telomerase activity as described in the supplier's manual.

Calculation of telomerase activity and statistics. Relative telomerase activities (RTA) within different samples in an experiment were obtained employing the formula provided by the manufacturer. An internal DNA standard consisting of 216-base pairs was included for the detection of Taq DNA polymerase inhibitors. The internal DNA standard also served as a reference for quantitation of telomerase activity. Negative controls were included to assess specificity for telomerase. These controls were prepared by heat inactivation of cell extracts for 10 minutes at 85°C before assaying for telomerase activity. Reactions were performed in triplicate and the mean values were taken to quantify telomerase activity. RTA obtained from vehicle-exposed cells was set to 100% and RTA of cells exposed to either PGA₂ or 2ME were expressed as a percentage of their controls. Statistical significance of differences was evaluated with ANOVA. P < 0.05 was regarded as statistically significant and indicated by an asterisk (*) on the graphs.

RESULTS

RTA of either PGA₂ or 2ME-exposed cells were determined after 16 h and expressed as percentages of vehicle-treated control cells. No signals were obtained from heat-inactivated negative controls. PGA₂ exposure led to a statistically significant decrease in the RTA of the three cancer cell lines (HeLa, WHCO3, MCF-7) studied when compared to vehicle-treated controls (P < 0.05) (Fig. 1). HeLa cells were shown to be more susceptible to the effects of PGA₂ when compared to the other two cancer cell lines. In contrast to the cancer cell lines, the RTA of the non-tumorigenic MCF-12A cell line was statistically significant increased after 16 h of exposure to PGA₂ (P < 0.00005) (Fig. 2). The RTA of HeLa, WHCO3 and MCF-12A cells were statistically significant decreased after 16 h of 2ME exposure when compared to vehicle-treated controls (P < 0.05) (Fig. 1 & 2). (Note the difference in scale when comparing results of these two figures). The influence of 2ME on the RTA of MCF-7 cells was statistically insignificant. WHCO3 cells were more susceptible to the effects of 2ME when compared to the other cell lines studied. P < 0.05 was regarded as statistically significant and indicated by an * on the graphs.

DISCUSSION

Telomeric (TRs) repeats and the tumor suppressor protein p53 can be regarded as guardians of the genome, since both are associated with DNA repair pathways and genomic integrity checkpoints. Telomere stabilisation in immortal cells can be achieved
**Cancer cell lines**

Fig. 1  RTA of either PGA2- or 2ME-exposed cancer cells after 16 h expressed as percentages of vehicle-treated control cells. No signals were obtained from heat-inactivated negative controls. Relative telomerase activities (RTA) within different samples in an experiment were obtained employing the formula provided by the manufacturer. Reactions were performed in triplicate and the mean values were taken to quantify telomerase activity. RTA obtained from vehicle-exposed cells was set to 100% and RTA of cells exposed to either PGA2 or 2ME were expressed as a percentage of their controls. $P < 0.05$ was regarded as statistically significant and indicated by an * on the graph.

**Non-tumurogenic cell line**

Fig. 2  RTA of either PGA2- or 2ME-exposed non-tumorigenic MCF-12A cells after 16 h expressed as percentages of vehicle-treated control cells. No signals were obtained from heat-inactivated negative controls. Relative telomerase activities (RTA) within different samples in an experiment were obtained employing the formula provided by the manufacturer. Reactions were performed in triplicate and the mean values were taken to quantify telomerase activity. RTA obtained from vehicle-exposed cells was set to 100% and RTA of cells exposed to either PGA2 or 2ME were expressed as a percentage of their controls. $P < 0.05$ was regarded as statistically significant and indicated by an * on the graph.
via the activation of the telomerase enzyme responsible for the assembly of TRs on telomeres (10). Genome instability is a key factor leading to the activation of the p53 and the subsequent growth arrest and/or apoptosis (1). p53-dependent growth arrest of cells may be the result of telomeric shortening occurring with aging. It was shown by (4) that growth arrest and apoptosis observed in telomerase-deficient mice were due to p53 activation. Critical shortening of telomeres or aberrations in their structure might be recognized as DNA damage by the p53-dependent cell-cycle checkpoint system (16).

Our previous research has shown that PGA2 and 2ME caused significant decreases in cell growth and subsequent induction of apoptosis in three cancer cell lines (HeLa, WHCO3 and MCF-7) (14, 15, 29, unpublished data). In HeLa cells, PGA2 reduced cell numbers significantly to 75% after 24 h and exposure of 48 h decreased cell numbers to 61% of the control. Growth inhibition in PGA2-treated MCF-7 cells was more pronounced than in MCF-12A cells (unpublished data). In MCF-7 cells, PGA2 significantly reduced cell numbers to 48% after 24 h and to 20% after 48 h, compared to vehicle-treated control cells. PGA2 decreased cell growth to 44.7% in WHCO3 after 48 h of exposure. The anti-mitogenic effects of PGA2 were confirmed by morphological studies where chromatin aggregation, cell membrane blebbing and uneven distribution of chromosomes were observed. Cell cycle progression analysis of HeLa and MCF-7 cells, showed an increase in DNA content preceding the G0/G1 peak after 48 h of exposure, which is indicative of apoptotic body formation (14).

Flow cytometry analysis of 2ME-treated HeLa and WHCO3 cells indicated increased cell numbers in G2/M. In WHCO3 cells, almost no G1 cells could be detected. The number of apoptotic cells also increased, due to apoptosis being induced in these cells unable to recover from the G2/M block (unpublished data). In both MCF-7 and HeLa cells, 2ME caused faulty spindle formation that resulted in a metaphase block and abnormal distribution of chromosomes (30). Hypercondensed chromosomes in arrested metaphase cells, indicative of apoptosis, as well as micronuclei formation were observed in 2ME-treated MCF-7 and HeLa cells (29). Growth inhibition in MCF-7 cells treated with 2ME was more pronounced than in MCF-12A cells (unpublished data).

A marked elevation in p53 levels was noticed in HeLa (10% increase) and WHCO3 cells (46% increase) after exposure to PGA2 (15). The latter suggests a possible p53-dependent apoptotic pathway in WHCO3 cells accompanied with a decrease in telomerase activity observed in this study. According to the literature, another cyclopentenone prostaglandin, PGA1, also caused inhibition of cell growth in three melanoma cell lines together with marked impairment of telomerase activity (8). Whether PGA2 activates a p53-dependent apoptotic pathway accompanied with a decrease in telomerase activity in HeLa cells, is unclear, since research has shown that the oncogenic human papilloma viruses are able to efficiently target p53 for degradation by the ubiquitin pathway (12). HPV-E6 proteins abolish p53 function by two distinct mechanisms that include the repression of p53 transcriptional activity by targeting the p53 coactivator CBP/p300 and the removal of cellular p53 protein through proteosome degradation pathway (32, 36).

In this study HeLa cells were shown to be more susceptible to the effects of PGA2 on the RTA when compared to the other two cancer cell lines. A decrease in the RTA of PGA2-treated MCF-7 cells was also observed. In contrast to the cancer cell lines investigated, PGA2 caused a marked increase in the telomerase activity of MCF-12A. The increased telomerase activity observed in the spontaneously immortalised MCF-12A cell line could possibly contribute to telomere stabilisation achieved via the activation of the telomerase enzyme. 2ME decreased the RTA of HeLa, WHCO3 and MCF-12A cells. WHCO3 cells were shown to be more susceptible to the effects of 2ME when compared to the other cell lines studied. The influence of 2ME on the RTA of MCF-7 cells was insignificant. The possibility exists that 2ME may exert its inhibitory effect on the RTA of MCF-7 cells after prolonged exposure, since literature has shown that inhibition of telomerase activity in cancer cells became more pronounced after prolonged exposure (8).

These results confirm that PGA2 and 2ME could be of potential value as anti-tumor agents, since both exert a direct cytostatic/cytotoxic effects on cancer cells and an inhibitory activity on the tumour-associated function of the telomerase enzyme involved in cancer cell immortality.

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