Growth Inhibition and Chromosomal Instability of Cultured Marsupial (Opossum) Cells after Treatment with DNA Polymerase α Inhibitor

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The DNA replication mechanism has been well established for eutherian mammals (placental mammals such as humans, mice, and cattle), but not, to date, for metatherian mammals (marsupials such as kangaroos, koalas, and opossums). In this study, we found that dehydroaltenusin, a selective inhibitor of mammalian (eutherian) DNA polymerase α, clearly suppressed the growth of metatherian (opossum and rat kangaroo) cultured cells. In cultured opossum (OK) cells, dehydroaltenusin also suppressed the progression of DNA replication. These results suggest that dehydroaltenusin inhibits metatherian as well as eutherian DNA replication. Dehydroaltenusin treatment of OK cells engendered fluctuations in the numbers of chromosomes in the OK cells as well as inhibition of cell growth and DNA replication. This suggests that partial inhibition of DNA replication by dehydroaltenusin causes chromosomal instability in cultured cells.

Key words: metatheria; opossum; DNA polymerase α; chromosomal instability; inhibitor

The DNA polymerase α (pol-α)-primase complex is an enzyme complex that is important to the initiation of eukaryotic DNA replication. It plays a central role in the synthesis of leading and lagging strands in higher eukaryotes.1) DNA replication begins when the primase subunit (p46 in humans, p48 in mice) of the pol-α-primase complex synthesizes a short RNA primer, followed by short DNA synthesis done by the catalytic subunit (p180 in both humans and mice) of pol-α.2,3) Eukaryotic pol-α has been investigated extensively in such diverse eukaryotes as Saccharomyces cerevisiae, Drosophila melanogaster, Xenopus laevis, Gallus gallus, and several mammals including Homo sapiens, Mus musculus (mice), and Bos taurus (calfes). Although pol-α has been well studied in eutherian mammals, no study of pol-α in metatherian or prototherian (monotremes such as platypuses) mammals has been reported to date. Recent genome analysis of an opossum gene (GenBank XM_001365137) homologous to the catalytic subunit (POLA1) of the eutherian pol-α gene. These sequence data show that the opossum pol-α catalytic subunit consists of 1,620 amino acid residues, significantly longer than that of the human homolog (1,462 a.a.). Comparison of these two amino acid sequences reveals that although the polymerase domains are conserved, the opossum pol-α catalytic subunit has a unique C-terminal region (data not shown).

Dehydroaltenusin is a selective inhibitor of mammalian pol-α isolated from a fungus, Alternaria tenuis, during screening for replicative DNA polymerase inhibitors.4,5) It is reportedly a strong inhibitor of mammalian pol-α, but it does not affect other DNA polymerases such as mammalian pol-δ, pol-ε, or pol-α from other vertebrates. Furthermore, dehydroaltenusin and its derivatives, such as C12, inhibit the growth of various cultured cells, including cancer cells.5–7) Although the effects of dehydroaltenusin on eutherian pol-α are well known, those on metatherian pol-α have not been studied to date. If dehydroaltenusin inhibits metatherian pol-α in addition to eutherian pol-α, it should be a useful tool for studying the structure and function of metatherian pol-α.

To determine whether dehydroaltenusin inhibits the growth of metatherian as well as eutherian cells in culture, we treated OK cells derived from opossum (Didelphis marsupialis virginianna) kidneys and PtK2 cells derived from rat kangaroo (Potorous tridactylus) kidneys with dehydroaltenusin. We found that dehydroaltenusin suppressed cell growth and the progression of DNA replication in these metatherians. Furthermore, our data suggest that treating OK cells with dehydroaltenusin causes chromosomal instability.

Materials and Methods

Materials. OK and PtK2 cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Biotin-16-dUTP was from Roche Diagnostics (Penzberg, Germany). Alexa Fluor 488-streptavidin was from Molecular Probes (Eugene, OR). Dehy-
droaltenusin was prepared by previously described chemical synthesis methods, \(^3\) and was purified to >99% purity.

**Cell culture and the effect of dehydroaltenusin on cell growth.** PtK2 and OK cells were cultured in 96-well plates at 37°C in minimal essential medium (MEM) containing non-essential amino acids, l-glutamine, and 10% fetal calf serum (FCS). Dehydroaltenusin was dissolved in dimethylsulfoxide (DMSO) and added to the cultured OK cells at the indicated concentrations. Control cells were treated with DMSO only (0 µM dehydroaltenusin). After the indicated durations, live cells were counted using a kit (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan) and a microplate reader (EL800; Central Kagaku-Boeki, Inc., Tokyo).

Visualization of sites of DNA replication. OK cells were cultured in 4-well chamber slides, and dehydroaltenusin was added in the indicated amounts for hypotonic shift assay, which was performed as previously described. \(^5\) Sites of DNA replication were detected by the incorporation of nucleotide analog biotin-16-dUTP into the cells after incubation in hypotonic buffer, as previously described. \(^12\)-\(^16\) The indicated amounts of dehydroaltenusin and biotin-16-dUTP were introduced into the cells simultaneously by hypotonic shift assay over 1 h. The cells were then incubated in fresh media for 30 min and fixed. The incorporated biotin-16-dUTP was visualized with Alexa Fluor 488-streptavidin, and signal-positive (replicating) cells were counted manually using a fluorescence microscope (BX50; Olympus, Tokyo).

Analysis of chromosomes. After 1 month of treatment with the indicated amounts of dehydroaltenusin (long-term exposure to dehydroaltenusin), OK cells were treated with 0.05 µg/mL of demecolcine solution for 2 h and then fixed, and the chromosomes were stained with 4′,6-diamidino-2-phenylindole (DAPI). The chromosomes of the fixed cells on the slide glass were visualized by fluorescence microscopy, and were counted manually.

**Results and Discussion**

The results of previous studies indicate that dehydroaltenusin inhibits the proliferation of various cultured eutherian cells, including human cancer cells. \(^5\) Based on these earlier studies, we used dehydroaltenusin, a specific, effective pol-α inhibitor, \(^5\) to inhibit pol-α function. Dehydroaltenusin inhibits eutherian pol-α in a dose-dependent manner. \(^5\) Thus, it can be a useful tool for titrating the amount of “active” pol-α in living cells. First we examined to determine whether dehydroaltenusin inhibits metatherian as well as eutherian cell proliferation. Two metatherian cell lines, OK and PtK2, were tested for growth inhibition by dehydroaltenusin. The OK cells were cultured as described above and treated with the indicated amounts of dehydroaltenusin for the indicated periods. For unknown reasons, the DMSO-treated (control) OK cells grew extremely rapidly, multiplying 28-fold in 1 d (data not shown). We believe that repeated passage of OK cells in the culture caused this rapid growth. OK cell growth was inhibited by the addition of dehydroaltenusin in a dose-dependent manner (Fig. 1), as was PtK2 cell growth (data not shown), but the inhibitory effect of dehydroaltenusin on PtK2 cells was less marked than on OK cells. It has been reported that dehydroaltenusin efficiently inhibited the growth of various cultured eutherian (human) cells in a dose-dependent manner. \(^6\) This suggests that dehydroaltenusin also inhibits metatherian cell growth. The LD\(_{50}\) values for inhibition of the growth of cultured human cells, such as HeLa, NUGC-3, and A549 cells, were 38, 43, and 44 µM respectively. These are much higher than those observed for OK cells in the present study (<10 µM, see also Fig. 1). This suggests that opossum OK cells are more sensitive to dehydroaltenusin than are human cells, but whether this enhanced sensitivity is common to all marsupial cells or unique to OK cells has yet to be established.

Based on previous reports \(^5,9,17\) and the data presented in Fig. 1, we believe that dehydroaltenusin suppresses metatherian cell growth via inhibition of pol-α. Hence we next examined to determine whether dehydroaltenusin inhibits the progression of DNA replication in metatherian cells. DNA replication in OK cells was monitored by visualization of incorporated biotin-16-dUTP, as described above. Compared with non-treated cells, the OK cells pre-treated with dehydroaltenusin for 2 d showed deceased DNA replication under biotin-16-dUTP incorporation (data not shown). Furthermore, the simultaneous addition of dehydroaltenusin and biotin-16-dUTP clearly suppressed DNA replication in the OK cells (Fig. 2). According to previous reports, dehydroaltenusin is thought to bind the catalytic subunit of pol-α and to inhibit pol-α activity. \(^5\) This suggests that dehydroaltenusin suppresses DNA replication by binding directly to the catalytic subunit of pol-α. We have not yet tested to determine whether dehydroaltenusin directly inhibits the enzymatic activity of opossum pol-α in a similar manner, because opossum pol-α has not yet been purified. For eutherian pol-α (bovine), it was suggested that dehydroaltenusin binds directly to the DNA template-primer binding site of the catalytic subunit. \(^5\) Because this DNA template-primer binding site is known to be located in the conserved polymerase domain of eutherian pol-α, \(^3\) we surmise that dehydroaltenusin also binds to the DNA binding site of opossum pol-α, despite the presence of the unique C-terminal region of pol-α. In the future, in vitro pol-α assays using purified native or recombinant pol-α must be done to test this hypothesis.

Lemoine et al. have reported that low levels of pol-α gene expression induced chromosomal translocation in *Saccharomyces cerevisiae*. \(^19\) Furthermore, they reported that reduced levels of pol-δ, an enzyme important for the elongation of lagging strands, induced chromosome fragile site instability in *Saccharomyces cerevisiae*. \(^19\) Hence we performed simple experiments to test the effects of dehydroaltenusin on the chromosomal stability...
of cultured OK cells. First, OK cells were subjected to prolonged treatment (approximately 1 month) with dehydroaltenusin. Each time the cells were passaged, dehydroaltenusin was added to fresh medium. At the end of the treatment period, the cells that had received long-term dehydroaltenusin treatment were arrested at M phase by the addition of demecolcine, and their chromosomes were prepared for DAPI staining and microscopic analysis as described above in Materials and Methods. Stained chromosomes were counted manually by fluorescence microscopy. The opossum Didelphis marsupialis virginiana has a normal diploid chromosome number of 22. OK cells established from Didelphis marsupialis virginiana show moderate mixoploidy, with 22, 23, or 24 chromosomes. Almost all of the OK cells used in this study had 24 chromosomes (Fig. 3A). Cell culture in the presence of dehydroaltenusin for 1 month caused fluctuations in the number of chromosomes in a dose-dependent manner (Table 1). After dehydroaltenusin treatment (especially at concentrations of 3.13 and 6.25 µM), the number of OK cells with a chromosome number other than 24 increased (Fig. 3B). This range of dehydroaltenusin concentrations (3.13–6.25 µM) was permissive for OK cell growth (Fig. 1). Figure 3B and Table 1 also show the occurrence of non-treated cells with 47–48 chromosomes, presumably caused by cell fusion during culture. These results suggest that inhibition of DNA replication by long-term dehydroaltenusin treatment causes the number of chromosomes in OK cells to fluctuate from generation to generation.

Partial inhibition of DNA replication causes gaps and breaks in metaphase chromosomes at common fragile sites (CFSs). Sister-chromatid exchange, translocation, and deletions are known to arise at these CFSs when cells are under replication stress. Treatment of

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**Fig. 2.** Inhibition of DNA Replication in Cultured OK Cells by Dehydroaltenusin.

A, Pre-cultured OK cells were incubated for 1 h in the absence and the presence of the indicated amounts of dehydroaltenusin (shown as hollow characters), followed by analysis of the progression of DNA replication measured by incorporated biotin-16-dUTP and visualized by fluorescence microscopy, as described in Materials and Methods. Light-spotted (green or light blue signal-positive) cell nuclei indicate that the DNA replicated and incorporated biotin-16-dUTP. Numbers within the figures signify the amounts of dehydroaltenusin. B, Total cells and signal-positive (replicating) cells were counted manually. White columns with numbers indicate the numbers of replicating cells. Gray columns indicate the numbers of non-replicating cells. Black lines signify the percentages of replicating cells. Data are represented as means and standard deviations for three experiments.

**Fig. 3.** Effect of Dehydroaltenusin on Chromosome Numbers of OK Cells.

A, The typical number of chromosomes in the OK cells cultured in our laboratory was 24. B, The number of chromosomes present in the OK cells after long-term (approximately 1 month) exposure to dehydroaltenusin (3.13 or 6.25 µM) fluctuated more than that in the non-exposed control cells. White columns indicate the numbers of chromosomes in non-exposed control cells; gray columns, the numbers of chromosomes in cells exposed to 3.13 µM dehydroaltenusin; and black columns, the numbers of chromosomes in cells exposed to 6.25 µM dehydroaltenusin. On the horizontal axis, the chromosome number range from 33 to 45 was omitted due to the absence of cells with chromosome numbers in this range. These data were obtained from a representative experiment among three independent experiments. The other two experiments showed similar fluctuations in chromosome numbers (data not shown).
cultured cells with aphidicolin, an effective inhibitor of pol-α and other polymerases, has been reported to cause chromosomal instability, particularly at fragile sites including CFSs.\textsuperscript{23,24} These previous pol-α inhibitor studies suggest that dehydroaltenusin causes fluctuations in the numbers of chromosomes in OK cells by affecting CFSs. In an analysis by Lemoine et al.,\textsuperscript{19} chromosomal translocation induced by a reduction of pol-α expression (to approximately 10% of the wild-type level) occurred between Ty elements, a yeast retrotransposon, on two chromosomes, probably due to delayed Okazaki fragment synthesis followed by the appearance of large single-stranded regions on the lagging strand, causing double-strand DNA breakage.\textsuperscript{10} We cannot confirm that the decrease in “active” pol-α molecules in this study was as drastic as the 90% reduction reported by Lemoine et al.\textsuperscript{19} We found that cell growth inhibition due to dehydroaltenusin, an effective pol-α inhibitor, caused fluctuations in the numbers of chromosomes in OK cells (Fig. 3B). In the future, we plan to analyze in detail how inhibition of pol-α induces chromosomal instability in OK cells with reference to CFSs such as FRA3B-homologous CFS in OK cell chromosomes.

At present, the relationship between the presence of the unique C-terminal region in the catalytic subunit of opossum pol-α and the increased sensitivity of OK cells to dehydroaltenusin relative to human cell lines is not clear. Furthermore, we cannot be certain that the observed fluctuations in the numbers of chromosomes in OK cells were a direct result of inhibition of pol-α activity. At present, we have no images of OK cell chromosomes directly showing dehydroaltenusin-mediated chromosomal instability, at CFSs or other sites. However, if pol-α inhibition is indeed responsible for these fluctuations in chromosome numbers, the results of this study are expected to provide insight into the molecular mechanism of pol-α in maintaining metatherian chromosomal stability, perhaps leading to future studies of both mammalian evolution and the molecular evolution of pol-α.

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