Basic Research Paper

Effects of cytochrome P450 arachidonic acid epoxygenases on the proliferation of tumor cells

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Background and Objective: Cytochrome P450 (CYP) arachidonic acid epoxygenases promote cell proliferation and inhibit apoptosis in endothelial cells. This study was to investigate the effects of CYP epoxygenases on the proliferation of tumor cells and possible signaling pathways. Methods: The effects of recombinant adenovirus (rAAV) mediated cytochrome P450 2J2 (CYP2J2), cytochrome P450 F87V (CYPF87V) and anti-CYP2J2 on proliferation of Tca-8113, A549, Ncl-H446 and HepG2 cells were measured using MTT and flow cytometry. Expressions of phosphorylated epidermal growth factor receptor (EGFR), extracellular signal-regulated kinase (ERK)1/2, PI3K, and Akt before and after transfection were detected by western blot. Tca-8113 cells infected with rAAV-CYP2J2, rAAV-CYPF87V, rAAV-antiCYP2J2 and rAAV-GFP were inoculated into nude mice, to observe the effect of CYP epoxygenases on the growth of xenografts. Results: Infection of Tca-8113, A549, Ncl-H446 and HepG2 cells with rAAV-CYP2J2 and rAAV-CYPF87V significantly increased the proliferation of tumor cells by 1.7-, 1.4-, 1.6- and 2.2-fold, and 2.0-, 1.5-, 1.8- and 2.0-fold respectively, as compared with control cells. On the contrary, infection with rAAV-antiCYP2J2 inhibited the proliferation of the four tumor cell lines. Moreover, CYP epoxygenases remarkably enhanced phosphorylation of EGFR, ERK1/2 and Akt, and upregulated total PI3K by 2-, 2.3-, 2.4- and 1.9-fold in the four cell lines, while rAAV-antiCYP2J2 exerted an inhibition effect. Infection of CYP450 epoxygenase genes markedly increased the cell percentage in S/G2/M phases by 210% as compared to control Tca-8113 cells. rAAV-CYP2J2 and rAAV-CYPF87V promoted tumor growth of Tca-8113 cell xenografts in nude mice in comparison to the control rAAV-antiCYP2J2 groups. Conclusion: CYP epoxygenases efficiently promote the proliferation of tumor cells, which may be related with the activation of EGFR, ERK1/2 and PI3K/Akt signaling pathways.

Materials and Methods

Agents and equipments. 17-ODYA, a CYP epoxygenase inhibitor, dissolved in anhydrous ethanol, 3-(4,5-dimethylthiazol)-2,5-diphenyl- tetrazoliumromide (MTT), propidium iodide (PI) and RNase A were purchased from Sigma Company (USA). Fetal bovine serum (FBS), DMEM, and trypsin were the products of Gibco Company (Germany). Anti-CYP2J2 and anti-CYPF87V antibodies were kindly provided by Dr. Capdevila of Vanderbilt University, USA. Anti-ERK1/2 and anti-phosphorylated ERK antibodies were purchased from New England Biolabs Ltd. (UK). Anti-Pi3K, anti-Akt, anti-phosphorylated Akt, anti-β-actin, and horseradish peroxidase-coupled goat anti-rabbit IgG antibodies were purchased from Santa Cruz (USA). Chemical fluorescent agent ECL was purchased from the Pierce Company (USA). rAAV-GFP, rAAV-anti-CYP2J2, rAAV-CYP2J2, and rAAV-CYPF87V were constructed and packaged at our laboratory. The remaining agents were manufactured and purified in China.

Cell culture. Lung cancer cell line A549, squamous cell carcinoma of the tongue Tca-8113, small-cell lung cancer cell line Ncl-H446, hepatic carcinoma cell line HepG2, fibroblast cell line HT-1080 and 293 cell line were purchased from the Institute of Biochemistry and Cell Biology in Shanghai (from American Type Culture Collection, ATCC, USA). Cell were cultured in DMEM containing 100u/ml penicillin and 100mg/L streptomycin, supplemented with 10% FBS at 37°C in a humidified atmosphere containing 5% CO₂.
Protein preparation and western blot. Tumor cells were plated in six-well plates, approximately \(3 \times 10^6\) cells per well. When the cells reached 60% confluence, viral solutions of rAAV-GFP, rAAV-CYP2J2, rAAV-antiCYP2J2, and rAAV-CYPF87V were added at 1000 vp/cell respectively, and incubated for 72 h. Cells were washed once with pre-cooled phosphate buffer (PBS), and 150 µl of cell lysis solution containing three detergents [50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 1.5 mmol/L MgCl2, 1 mmol/L EDTA, 1% Triton X-100, 10% glycerol, 10 µg/mL Leupeptin, 10 µg/mL aprotinin, 100 mmol/L NaF, and 200 µmol/L NaVO4] was added into each well. After incubation for 20 min on ice, cells were scraped and transferred to microcentrifuge tubes, centrifuged for 5 min at 5000 rpm and 4°C. Supernatant was then transferred to a new centrifuge tube. The protein concentration in the cell lysis solution was measured by the Bradford method. The expressions of proteins CYP2J2 and CYPF87V were measured by western blot.4

MTT assay. One day prior to transfection, cells were plated in six-well plates, approximately \(3 \times 10^6\) cells per well. When the cells reached 60% confluence, the viral solutions of rAAV-GFP, rAAV-antiCYP2J2, rAAV-CYP2J2 and rAAV-CYPF87V at 1000 vp/cell were added respectively, and cultured for 72 h. Then cells were washed with PBS (pH7.4), digested by 0.25% trypsin. Re-suspension of single cell was prepared at a density of \(5 \times 10^3\) cells per well, and inoculated onto a 96-well plate. Cells were cultured at 37°C for 24 h in a humidified atmosphere containing 5% CO₂. Culture medium was discarded and MTT was added. The absorption value (A) was measured at the 490 nm wavelength. Cell proliferation rate was calculated as follows: cell proliferation rate = (A value of the experimental group / A value of the control group) × 100%.

Cell counting. Tca-8113, A549, Ncl-H446, HepG2 and HT-1080 and 293 cell lines in a good condition were harvested and digested by 0.25% trypsin. Suspension of single-cell was prepared using DMEM with 10% FBS. Cells were inoculated onto a 24-well plate at a density of \(1 \times 10^4\) cells per well. After 24 h culture, culture medium was discarded and free DMEM was added. Cells were incubated for another 12 h for synchronization, vehicle (anhydrous ethanol) and 17-ODYA (a final concentration of 100 µmol/l) were added, and cultured for another 24 h. Blood counter was used to count the cell number of each well.

Measurement of cell cycle by flow cytometry. After 72 h transfection with cytochrome P450 epoxygenases mediated by recombinant adeno-associated virus (AAV), Tca-8113 cells were routinely digested and centrifuged. Cells were washed twice with PBS and centrifuged for 5 min at 2000 rpm. Supernatant was discarded and cells were re-suspended in 1 ml ice-cold saline. One milliliter of 75% ice ethanol was added for fixation. Cells were shaken for 30 min and centrifuged again for 5 min at 2000 rpm. Then the supernatant was discarded. Cells were filtered and washed three times with PBS, followed by RNase digestion for 30 min, addition of 1 ml PI (0.05 g/L) to adjust the cell density to \(1 \times 10^8\) cells/ml. Staining was performed in dark at 4°C for 15 min. Samples were loaded and measured by flow cytometry at the wavelength of 488 nm.

Measurement of signal transduction molecules. Cells were plated in a six-well plate, approximately \(3 \times 10^6\) cells/well. When the cells grew to 60% confluence, viral solutions of rAAV-GFP, rAAV-CYP2J2, rAAV-antiCYP2J2 and rAAV-CYPF87V at 1000 vp/cell were added respectively, and incubated for 72 h. After being washed by pre-cooled PBS, 150 µl cell lysis solution was added into the cells. After 20 minutes of ice bath, cells were scraped and transfected to microcentrifuge tubes. Cells were centrifuged at 5000 rpm and 4°C for 5 min. The supernatant was transferred to a new centrifuge tube. The protein concentration in the supernatant was measured using the Bradford method. Expressions of signal molecules EGFR, Akt, phosphorylated ERK and protein P13K were measured using western blot.

Establishment of xenograft tumor models in nude mice. Thirty nude mice, around four to six week old (an average weight of 20.5 g), with unspecified sex, were selected and randomly divided into five groups, six in each group. Tca-8113 cells transfected with different genes were inoculated in the armpit of the right limb of each mouse (\(5 \times 10^6\) cells/mouse). The location of inoculation was observed daily, including swelling, ulceration, formation of the subcutaneous tumor and the status. On the days 7, 12, 17, 22, 27 and 32 day after subcutaneous inoculation, the longest diameter (a) and the shortest diameter (b) of the tumor mass were measured. The volume of the tumor mass was \(V = \frac{a \times b^2}{6}\), and the growth curve of the tumor mass was plotted. Animals were euthanized on day 32 after inoculation. The tumor tissues were isolated and frozen at -80°C. Expressions of CYP epoxygenases in tumor tissues were measured by western blot.

Statistical analyses. All data are presented as mean ± SE. SPSS 13.0 software was used for variance analysis of multiple samples, and t-test for the analysis of differences in two groups. p < 0.05 is regarded as statistically significant.

Results

Expression of CYP epoxygenase genes after transfection in tumor cells. Tca-8113 cells were transfected with rAAV-GFP, rAAV-CYP2J2 and rAAV-CYPF87V. After 72 h, protein expressions of CYP2J2 and CYPF87V were remarkably elevated in cells transfected with CYP2J2 and CYPF87V, while the expression of CYP epoxygenases remained unchanged in the rAAV-GFP group and 17-ODYA group. The expression of CYP2J2 was significantly weaker in the rAAV-antiCYP2J2 group than in the non-transfected (control) group (Fig. 1).

Influence of CYP epoxygenases on the proliferation of four tumor cell lines. In comparison with the control and the rAAV-GFP groups, transfection of rAAV-CYP2J2 and rAAV-CYPF87V significantly promoted the proliferation of tumor cells by 1.4- to 2.2-fold, which were a 1.7- to 2.0-fold increase in A549 cells, a 1.4- to 1.5-fold increase in Tca-8113 cells, a 1.6- to 1.8-fold increase in HepG2 cells, and a 2.2- and 2.0-fold increase Ncl-H446 cells (p < 0.01). After transfection with rAAV-antiCYP2J2 and addition of 17-ODYA, cell proliferation was reduced more than 50% compared with the controls (p < 0.01) (Fig. 2).

Influence of a CYP epoxygenase inhibitor, 17-ODYA, on tumor cell proliferation. Strong expression of CYP2J2 was detected in four tumor cell lines, A549, Tca-8113, Ncl-H446 and HepG2, while no expression of CYP2J2 was observed in control HT-1080 and HEK293 cells (Fig. 3A). 17-ODYA significantly inhibited proliferation of tumor cells expressing CYP2J2 in a dose-dependent manner, with a maximum rate of 55%. However, 17-ODYA had no apparent inhibition effects on cell proliferation of cells without expression of CYP epoxygenase genes. (Fig. 3B).
Effects of cytochrome P450 arachidonic acid epoxygenases on the proliferation of tumor cells

Effect of different treatments on cell cycle progression in Tca-8113 cells. The ratios of Tca-8113 cells transfected with rAAV-CYP at the S- and G2-M phases were significantly higher than those of the control group (p < 0.01), increased by 210% in total. Meanwhile, cells treated with 17-ODYA and transfected with rAAV-antiCYP2J2 were significantly increased in G1-phase, while decreased by 43.7% in S-phase, in comparison to the control and the rAAV-GFP groups (p < 0.05) (Fig. 4).

Influence of CYP epoxygenases on expressions of EGFR, ERK1/2, and PI3K/Akt. The protein levels of phosphorylated EGFR, ERK1/2, and Akt, and total PI3K were significantly higher in Tca-8113 cells transfected with CYP2J2 and CYPF87V than in control cells, which were increased by 2- to 2.4-fold, and 1.8- to 2.4-fold, respectively (p < 0.01). In the mean time, the protein levels of phosphorylated EGFR, ERK1/2, and Akt, and total PI3K were reduced by 53%, 40%, 64% and 43% in Tca-8113 cells transfected with rAAV-antiCYP2J2, and were reduced by 42%, 63%, 42% and 34% respectively in Tca-8113 cells treated with 17-ODYA compared with the control groups. The differences were statistically significant (p < 0.05) (Fig. 5).

Transfection of CYP epoxygenase genes on the growth of Tca-8113 cell xenografts in nude mice. All 30 nude mice survived, among which 28 were formed a single subcutaneous tumor. Tca-8113 cells transfected with rAAV-antiCYP2J2 did not form tumors in two mice. All tumors were pathologically confirmed as the transplanted tumor. The protein levels of CYP2J2 and CYPF87V were significantly increased in the tumor tissues translated with Tca-8113 cells transfected with rAAV-CYP2J2 and rAAV-CYPF87V, while were decreased in those transfected with rAAV-antiCYP2J2, in comparison to the control groups (Fig. 6). After inoculation of Tca-8113 cells with different treatments to nude mice for 4 to 7 days, subcutaneous nodules, observable to naked eyes, appeared. The transplanted tumor appeared earlier in the rAAV-CYP2J2 (5.8 d) and rAAV-CYPF87V (6.0 d) groups than in the control (8.4 d), rAAV-GFP (8.6 d), and rAAV-antiCYP2J2 (10.0 d) groups. The tumors grew rapidly, and reached the peaks around 12 to 22 days. The tumor growth was mostly delayed in the rAAV-antiCYP2J2 group, and the difference was most apparent on day 17. On the contrary, the tumors in the rAAV-CYP2J2 group showed rapid growth, reached the peak on day 12, and remained rapid growth even on day 32. The size of the tumors in the rAAV-CYP2J2 group was twice the size of those in the control group (Fig. 7).
Effects of cytochrome P450 arachidonic acid epoxygenases on the proliferation of tumor cells

Discussion

Cyclooxygenase and lipoxygenase pathways of arachidonic acid (AA) are widely studied and recognized. They are believed to be in close relation to the incidence and progression of tumors.6-9 Study on the CYP metabolic pathway of AA has become more important.

AA produces epoxyeicosatrienoic acids (EETs), which is also known as arachidonic acid epoxides, through CYP. Because EETs are relatively instable, they are converted into dihydroxepoxicosatrienoic acids (DHETs) by soluble epoxide hydrolase (sEH). Many extrinsic and intrinsic epoxides are believed to be potential carcinogens.10,11 CYP2J2 is highly expressed in myocardial cells and coronary artery,12 as well as in many other tissues, including lung, liver, kidney, jejunum, ileum, colon, stomach, pancreas, and pituitary gland.13 Recent studies show that CYP2J2 expression in malignant solid tumors is significantly higher than that in the surrounding and normal tissues.3,4 This research selected recombinant AAV, with high transfection efficiency and low adverse reaction, as the carrier to mediate the transfection of CYP epoxygenase genes into four different tumor cells. The CYP epoxygenase genes were actively expressed in tumor cells.

Figure 4. Cell cycle progression in Tca-8113 cells after different treatments.

Figure 5. Effects of CYP450 epoxygenase gene transfection on the expression of EGFR, ERK1/2, PI3K/Akt in TCA-8113 cells. *p < 0.05 vs. control group; **p < 0.01 vs. control group; ***p < 0.05 vs. rAAV-2J2 group.
used 17-ODYA to indirectly study the effect of CYP epoxygenases in tumor cells. We found that CYP epoxygenases significantly increased DNA synthesis in tumor cells, as well as cell division and proliferation. CYP2J2 can effectively metabolize AA to produce four types of EETs, among which 14,15-EET is the major one. CYPF87V is the mutant of bacterial CYP BM3 after the phenylalanine residue at position 87 is replaced by valine. CYPF87V can also effectively metabolize AA to produce 14,15-EET. We speculate that 14,15-EET plays a key role in promoting tumor cell proliferation induced by CYP2J2. Whether other metabolic products of CYP2J2, such as 5,6-EET, 8,9-EET, and 11,12-EET, also exert similar effects requires further investigation.

EETs induced by CYP are closely related to the regulation of intercellular signal transduction, cell proliferation, and inflammatory response. We explored the statuses of signal molecules, such as EGFR, PI3K, Akt, and ERK1/2, and so on, in tumor cells after transfection with CYP genes. The levels of phosphorylated EGFR, PI3K/Akt, and ERK1/2 in tumor cells transfected with cytochrome epoxygenases were significantly upregulated, while the total expressions of EGFR, Akt, and ERK remained unchanged. In addition, the levels of phosphorylated EGFR, PI3K/Akt, and ERK1/2 in cells transfected with antisense CYP2J2 and 17-ODYA were remarkably reduced. These results suggest that PI3K/Akt and ERK1/2 pathways are involved in the promotion of tumor cells by CYP epoxygenases.

In summary, cytochrome epoxygenases promote tumor cell proliferation, and the underlying mechanism involves several signal transduction pathways, such as EGFR, ERK, Akt, and so on. This study helps understand the genesis and progression of tumors, and may provide foundation for the prevention and treatment of tumors.

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**References**


**Effects of cytochrome P450 arachidonic acid epoxygenases on the proliferation of tumor cells**

**Figure 6.** Protein expressions of CYP2J2 and CYPF87V in Tca-8113 xenograft tissues in nude mice.

**Figure 7.** Effects of CYP450 epoxygenase gene transfection on the tumor growth of Tca-8113 cell xenografts in nude mice. p < 0.05 vs. control group; bp < 0.01 vs. control group; cp < 0.05 vs. rAAV-2J2 group.

Cells after transfection, confirming the reliability of rAAV infection. The proliferation of tumor cells was greatly promoted with the transfection of rAAV-CYP2J2 and rAAV-CYPF87V, but was significantly inhibited by rAAV-antiCYP2J2 and addition of 17-ODYA. 17-ODYA could inhibit the activity of CYP epoxygenases, thus cause a decrease of synthesis of intrinsic EETs.

Expressions of cytochrome epoxygenases are found to be cell- and tissue-specific. For example, CYP2J2 is widely distributed in heart, liver, lung, kidney, intestines, pancreas, and blood vessels with different intensities, especially abundant in heart and vascular endothelium. In order to prove the inhibition effect of CYP epoxygenases on various tumor cells, we selected four tumor cell lines originated from different origins in this study. We found that CYP epoxygenases promoted cell proliferation in four tumor cells, regardless of the cell of origin. In later experiments in vivo, we investigated the effect of CYP epoxygenases on the growth of Tca-8113 cell xenografts in nude mice. Results revealed that CYP epoxygenases significantly promoted the growth of transplanted tumors, which confirms the promotional effect of CYP epoxygenases on tumor cell proliferation.

17-ODYA, an inhibitor of CYP epoxygenases, can greatly inhibit the enzyme activity and reduce the synthesis of EETs. We used 17-ODYA to indirectly study the effect of CYP epoxygenases on tumor cell proliferation. In order to eliminate the cytotoxicity of 17-ODYA, we selected two cell strains without the expression of CYP epoxygenases, 293 and HT-1080 in the study. 17-ODYA dramatically inhibited proliferation of tumor cells expressing CYP epoxygenases in a dose-dependent manner, and the maximum inhibitory rate reached 55%. However, 17-ODYA did not reduce the proliferation of cells without the expression of CYP epoxygenase genes. This suggests that 17-ODYA achieves its inhibitory effect through inhibiting the activity of epoxygenases, but not via its cytotoxicity. This also reveals that the promotional effect of CYP epoxygenases on tumor cells is through metabolizing AA to produce EETs, but not directly by themselves. Moreover, CYP2J2 epoxygenases significantly increased DNA synthesis in tumor cells, as well as cell division and proliferation. CYP2J2 can effectively metabolize AA to produce four types of EETs, among which 14,15-EET is the major one. CYPF87V is the mutant of bacterial CYP BM3 after the phenylalanine residue at position 87 is replaced by valine. CYPF87V can also effectively metabolize AA to produce 14,15-EET. We speculate that 14,15-EET plays a key role in promoting tumor cell proliferation induced by CYP2J2. Whether other metabolic products of CYP2J2, such as 5,6-EET, 8,9-EET, and 11,12-EET, also exert similar effects requires further investigation.
Effects of cytochrome P450 arachidonic acid epoxygenases on the proliferation of tumor cells


