Abstract. Resistance to chemotherapy is very important in the prognosis of tumors. Transglutaminase-2 (TG-2) mediated chemotherapy resistance has been widely reported. The objective of this study was to demonstrate the effect of 4-hexylresorcinol (4-HR) on TG-2 activity in nasopharyngeal squamous cell carcinoma cells (KB cells). Treatment with a mixture of 4-HR and cisplatin significantly decreased KB cell viability compared to treatment by cisplatin alone at 10 µg/ml (p<0.001). 4-HR inhibited TG-2 activity compared to cisplatin alone at 5, 10 and 20 µg/ml (p=0.001, 0.001 and 0.003, respectively). Nuclear translocation of TG-2 was also inhibited by 4-HR treatment. 4-HR treatment also increased the fluorescence life-time of DAPI significantly compared to the untreated control or the cisplatin treated group (p<0.001). In conclusion, 4-HR inhibited TG-2 activity and showed a synergistic effect on tumor cell growth inhibition with cisplatin.

Introduction

Chemotherapy has been done pre- and/ or post-operatively for the treatment of advanced head and neck cancer. However, some head and neck cancers are resistant to chemotherapy via activated nuclear factor-κ B (NF-κB) (1). In addition, complications after cisplatin containing regimens increase according to the concentration of cisplatin (2). Moreover, it has been shown that there are equal survival rates for advanced head and neck cancers when comparing concurrently chemoradiation therapies to surgical resection (3). Therefore, the development of a new agent that can decrease the complications of chemotherapy and overcome the resistance that conventional chemotherapeutic agents have is necessary.

Some tumor cells can evade the apoptosis pathway induced by chemotherapy. Transglutaminase-2 (TG-2) is an enzyme with diverse function and may be related to the resistance of chemotherapy agents. TG-2 is overexpressed in many cancers such as breast cancer (4), malignant melanoma (5), and glioblastoma (6). The protein cross-linking activity by TG-2 is related to the resistance of chemotherapy agents (7). It is part of tumor biology because it is involved in the polymerization of the inhibitor of NF-κB (I-κB) (7). The polymerization of I-κB can produce many free forms of NF-κB. Therefore, the NF-κB downstream pathway can be activated, and this mechanism is related to tumor cells evading the apoptosis pathway. A tumor showing resistance to cisplatin also has active TG-2 (5,8). Therefore, a TG-2 inhibitor may increase tumor cell death when it is used with cisplatin.

4-Hexylresorcinol (4-HR) is a well known food additive (9). It is a pseudo-tyrosine-like chemical and strong inhibitor of tyrosinase (10). It can inhibit the growth of microorganisms (11). It was used in humans as oral medication in the 1920s for the treatment of typhoid infections (12). Now, it is also the ingredient of oral anti-septics. Oral intake of 4-HR can prevent some types of tumor occurrence in experimental animals (13). The mechanism of tumor inhibition by 4-HR is largely unknown.

In this study, the effect of 4-HR on TG-2 expression in KB cells by fluorescent immunocytochemistry and real-time RT-PCR is presented. The combined effect of cisplatin and 4-HR on KB cell survival was evaluated by MTT assay. The effect of 4-HR on TG-2 activity was evaluated by a commercially available enzyme assay kit. The change of nuclear translocation of transcription factor after administration of 4-HR was evaluated by fluorescence lifetime imaging.

Materials and methods

Cell cultures and MTT assay. KB cells (human nasopharyngeal carcinoma; ATCC: Manassas, VA, USA) were grown
TG-2 enzyme assay. TG-2 was purchased from Sino Biological Inc. (Beijing, China). The TG inhibition assay was done by transglutaminase assay kit (CAT#CS1070; Sigma). A sample (25 µl) containing 1-20 µg/ml of 4-HR or cisplatin was added to a test tube and the sample volume was adjusted to 50 µl with enzyme. The mixture was pre-incubated for 5 min. Each assay mixture was transferred to a single well on a plate. The mixture was incubated on ice for 1-2 h or at room temperature for 15-30 min. While incubating, 0.1 µl of streptavidin-peroxidase solution was diluted into 100 µl of phosphate-buffered saline Tween-20 (PBS-T), and this was done for each well. The wells were washed 3 times with ultrapure water. Freshly prepared streptavidin-peroxidase conjugate (100 µl) was added to each well and incubated for 20 min at room temperature. The wells were washed no more than 3 times with 200 µl of PBS-T for each well. 3,3',5,5'-Tetramethylbenzidine (TMB) Liquid Substrate System (200 µl) was added to each well. The samples were incubated for 1-3 min at room temperature. The color development was stopped by the addition of 100 µl of stop solution to each well. The absorption was read at 450 nm on a plate reader.

Fluorescence immunocytochemistry and real-time polymerase chain reaction (RT-PCR). The antibody for TG-2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) for fluorescence immunocytochemistry analysis. Fluorescence immunocytochemistry was done using a previously published method (14). 4,6-Diamidino-2-phenylindole (DAPI) was used for count staining.

For the RT-PCR analysis, KB cells were treated for 12 h with 4-HR or cisplatin (5 and 10 µg/ml). Total RNA (1 µg) was used as a template for first strand cDNA synthesis using the ImProm II Reverse Transcription System (Promega, Madison, WI, USA). RT-PCR for cisplatin treated KB cells was also done using the same method. RT-PCR protocol for TG-1 and TG-2 were done as previously described (15). The primer for TG-1 was GAGAGACCATCCCTATGGCA and GGCAATGTCCTTGCTCATCT (88 bp). The primer for TG-2 was AGCGGACTATCCCCATATGGCA and GCCATGTCCCTTGCTCATCT (105 bp).

Results

Synergistic effect of 4-HR and cisplatin on KB cell inhibition. Cell viability of KB cells was tested with cisplatin and 4-HR (Sigma, St. Louis, MO) were mixed together in equal amounts and added to confluent cells to final concentrations of 1, 5, or 10 µg/ml. Cisplatin and 4-HR (Sigma, St. Louis, MO) were mixed together in equal amounts and added to confluent cells to final concentrations of 1, 5, or 10 µg/ml.

Quantification of cell viability was assessed after 48 h of culture growth by tetrazolium salt 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were incubated with MTT solution (Cell proliferation kit I; Roche Molecular Biochemicals, Mannheim, Germany) in 6-well plates for 4 h at room temperature. The color development was stopped by the addition of 100 µl of stop solution to each well. The samples were incubated for 1-3 min at room temperature. The color development was stopped by the addition of 100 µl of stop solution to each well. The absorption was read at 450 nm on a plate reader.

Fluorescence lifetime imaging. Fluorescence lifetime imaging was done using an inverted-type scanning confocal microscope (MicroTime-200, Picoquant, Germany) with a x100 objective (NA = 1.4). A single-mode pulsed diode laser (375 nm with an instrumental response function (IRF) of ~240 ps in full-width at half maximum and an average power <1 µW) was used as an excitation source. A 50-µm pinhole and a bandpass filter of 460 nm (Thorlabs, FB460-10, Newton, NJ, USA) were used to collect emissions from the DAPI stained specimens which were put on a glass cover-slip. The time-resolved fluorescence information was obtained with a time-correlated single-photon counting (TCSPC) technique. The exponential fittings were reformed by an iterative least-squares deconvolution fitting routine. All lifetime values shown in this study were mean intensity-averaged lifetime (τint). Fluorescence intensity images without lifetime information were simply calculated using the Symphotime software (version 5.1) from the fluorescence lifetime images.

Figure 1. The results of the MTT assay. KB cells were treated for 48 h by cisplatin or cisplatin + 4-hexylresorcinol (4-HR).
cisplatin, respectively. The expression of the TG-2 gene was 0.80±0.03 and 0.91±0.06 after the administration of 5 and 10 µg/ml of cisplatin, respectively (Fig. 2B). Cisplatin did not inhibit TG-2 activity at the tested concentrations (1-20 µg/ml). However, 4-HR inhibited TG-2 activity in a dose-dependent manner (1-20 µg/ml). When comparing the cisplatin treatment and 4-HR treatment, the difference was statistically significant at 5, 10, and 20 µg/ml (p=0.001, 0.001, and 0.003, respectively).

Discussion

TG-2 facilitates tumor spread and metastasis (16). For example, TG-2 is involved in peritoneal spread of ovarian tumor (17). TG-2 mediated chemotherapy resistance has already been reported (18). TG-2 could be involved in apoptosis in which TG2-dependent cross-linking following increases in intracellular Ca2+ may be important in the stabilization of apoptotic...
Table I. Fluorescence lifetime of the cells including DAPI.

<table>
<thead>
<tr>
<th>Samples</th>
<th>$\tau_1$ (%)</th>
<th>$\tau_2$ (%)</th>
<th>$&lt;\tau_{int}&gt;$</th>
<th>$\chi^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.47±0.07 (44)</td>
<td>2.23±0.03 (56)</td>
<td>1.99±0.08</td>
<td>1.13</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0.27±0.04 (49)</td>
<td>2.24±0.03 (51)</td>
<td>2.03±0.03</td>
<td>1.16</td>
</tr>
<tr>
<td>4-HR</td>
<td>0.28±0.03 (32)</td>
<td>2.46±0.07 (68)</td>
<td>2.35±0.02</td>
<td>1.15</td>
</tr>
</tbody>
</table>

$I(t) = A_1e^{-t/\tau_1} + A_2e^{-t/\tau_2}$, where $I(t)$ is the time-dependent fluorescence intensity, $A$ is the amplitude (noted as the normalized percentage in the parenthesis), and $\tau$ is the lifetime. The excitation and the detection wavelengths are 375 and 460±5 nm, respectively. Intensity weighted average lifetime, $<\tau_{int}>$, is defined as $<\tau_{int}> = \frac{\sum \tau_i A_i}{\sum A_i}$.

Figure 5. The fluorescence lifetime of DAPI in the nucleus was significantly different in the 4-HR treated groups. The lifetime of the control (A) and that of cisplatin (B) were not different and statistically significant ($p>0.05$). However, the lifetime of the 4-HR treated group (C) was different and statistically significant to that of the untreated control or cisplatin alone treated groups ($p<0.001$).

Figure 6. Fluorescence lifetime decays of DAPI treated cells. An instrumental response function (IRF) with a width of ~240 ps in full-width at half maximum was also presented.

Figure 7. Probable mechanism of 4-HR on TG-2 activity and NF-κB pathway.
cells by preventing the loss of intracellular components (19). TG-2 protects ovarian cancer cells from cisplatin induced apoptosis (8). In this study, 4-HR inhibited the TG-2 enzyme activity (Fig. 3). When comparing the mixture containing 5 μg/ml 4-HR and 5 μg/ml cisplatin to 10 μg/ml of cisplatin alone, the mixture group had a significantly lower tumor cell viability than the cisplatin group (p<0.05). According to these findings, 4-HR had synergistic effects along with cisplatin on KB cell death.

TG-2 localizes mainly in the cytoplasm, yet recent reports also suggest its presence in the nucleus, mitochondria, at the cell surface, and in the extracellular matrix (20). Nuclear localization of TG-2 is associated with high levels of increased TG activity (21). TG-2 exhibited nuclear translocation in the untreated control (Fig. 4A). 4-HR inhibited nuclear translocation of TG-2 in the KB cells (Fig. 4B). Since 4-HR inhibited TG-2 activity, its nuclear localization may also be inhibited accordingly (Fig. 7). Since 4-HR did not inhibit TG-2 gene expression in KB cells, the inhibition mechanism of 4-HR on TG-2 might be due to direct binding of 4-HR to the TG-2 protein (Fig. 7). Interestingly, 4-HR can bind to tyrosinase directly and inhibits enzyme activity (10).

Pre-incubation with a specific NF-κB inhibitor blocks NF-κB nuclear translocation and reduces glutamate-increased TG-2 expression (22). TG-mediated I-κBα polymerization can activate NF-κB directly (23). This agrees with recent reports focusing on the role of TG-2 in preventing cell death depending on the kind of stimuli and cell type (24). NF-κB binds to AT-rich regions of the promoter in the nucleus (25). DAPI also binds dominantly to AT-rich regions of the DNA in the nucleus (26). If the amount of nuclear NF-κB is decreased, many AT-rich regions may be available for DAPI binding (Fig. 7). TG-2 is colocalized with the p65 subunit of NF-κB in the cytoplasm, and there is increased nuclear translocation of the p65/TG-2 complex in response to TG-2 activation (27). However, 4-HR may inhibit NF-κB nuclear translocation via TG-2 inhibition. Actually, 4-HR inhibited TG-2 nuclear translocation (Fig. 4). Then, it may have an influence on DAPI binding to AT-rich regions. In the control and cisplatin treated group, the fluorescence lifetime of DAPI in the nucleus was not significantly different (p>0.05). However, in the 4-HR treated group, the fluorescence lifetime of DAPI in the nucleus was significant increased compared to the others (p<0.001; Fig. 5). The elongated average lifetime was attributed to the reduced contribution of the shorter lifetime component as well as the elongation and increased contribution of the longer lifetime component (Table 1). According to previous studies, a short lifetime component (0.2 < τ < 0.6 ns) was assigned to a proton transfer in the excited state of the DAPI probe, while a long lifetime component (2.0 < τ < 4.0 ns) originated from a tightly bound DAPI-DNA complex (28,29). A relative contribution of a short lifetime component is, therefore, very useful to monitor solvent exposure of a DAPI-DNA complex. From this fluorescence lifetime imaging study, hence, it was considered that the 4-HR treated group was not much affected by the extra-molecular couplings which were plausibly triggered by intruded molecules through a translocation process. If NF-κB can not translocate into the nucleus, free AT-rich regions of DNA in the nucleus may bind to DAPI. Consequently, the fluorescence lifetime of DAPI in the nucleus increased in 4-HR treated groups (Figs. 5 and 6).

In conclusion, 4-HR had a synergistic effect when mixed with cisplatin on KB cells. Since 4-HR is a well known additive for food and has been taken by humans as a medicine in past, its safety to humans is known. Therefore, 4-HR could be a candidate as a chemotherapeutic agent for cisplatin resistant tumors. However, the results of the in vitro study can not be directly extrapolated to clinical applications. Further study must be encouraged for clinical applications.

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References


