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ENHANCED GREEN FLUORESCENCE PROTEIN TRACKS TRICHOSANTHIN IN HUMAN CHORIOCARCINOMA CELLS AS A FEASIBLE AND STABLE REPORTER

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1. ABSTRACT

Trichosanthin (TCS) is a ribosome-inactivating protein (RIP) which can inhibit the growth of human choriocarcinoma (JAR) cells. There are no clear mechanisms to discover the interaction pathway and cytotoxicity of TCS in JAR cells. In this paper, we showed the distribution and transport of endogenously expressed TCS in JAR cells. Enhanced Green Fluorescence Protein (EGFP), fused with TCS, was applied as a reporter to track the behavior of TCS in JAR cells. Firstly, we investigated the expression stability of EGFP and physiological effects on JAR cells. A stable cell line expressing EGFP was created, which could reproduce and express EGFP even if transplanted into nude mice. Based on the proved stability and feasibility of EGFP in cultured cells and *in vivo*, the fusion gene of EGFP and TCS was constructed and transfected into JAR cells by liposome. The fluorescence microscopy showed that TCS-EGFP fusion gene was expressed in JAR cells in 24 to 48 hours and the fluorescence spread in cytoplasm mainly and in nucleus partially, which could trace the distribution and transport of TCS-EGFP in JAR cells. Most of fluorescent cells died after 48 hours for the cytotoxicity of expressed TCS-EGFP. These results first reported a stable expression and tracing method by EGFP in JAR cells, and provided theoretical basis to apply TCS in cancer therapy.

2. INTRODUCTION

Human choriocarcinoma (JAR) cells are epithelial-like cells established from the trophoblastic tumor of the placenta of a 24-year-old Caucasian woman (1). This malignant cancer endangers many women all over the world. During the investigations to cure this disease, the researchers found an effective toxin to JAR cells, Trichosanthin (TCS), in *Trichosanthes kirilowii* Maxim (2). TCS is a type-I ribosome-inactivating protein (RIP) with a single polypeptide chain, and possesses multiple biological activities including anti-tumor and anti-HIV functions in clinic trials (3, 4). Especially, TCS has cytostatic activities to JAR cells (5), which involves the inactivation of ribosome and the inhibition of protein synthesis, and the caspase-related apoptosis (6, 7). JAR is an outstanding model to research these activities, intracellular transport and interaction pathway of TCS (8).

In order to track the behavior of TCS in JAR cells, a feasible and stable reporter is required. The green fluorescent protein (GFP), cloned from bioluminescent jellyfish *Aequorea victoria*, has been established as a novel genetic reporter system (9, 10). Light-stimulated GFP fluorescence is species-independent and does not require any cofactors, substrates, or additional gene products from

A. victoria (10). Additionally, detection of GFP and its variants can be performed in living cells and tissues as well as fixed samples. The red-shifted green fluorescent variant, enhanced green fluorescent protein (EGFP), has been developed, and the double-amino-acid substitutions of Phe-64 to Leu and Ser-65 to Thr cause that EGFP fluoresces 35-fold more brightly than GFP when excited with blue light (11). The bright fluorescence endues EGFP with high detection sensitivity in analyses including flow cytometry and microscopy.

However, no reports show the stability and applicability to express EGFP and toxin-EGFP fusion proteins in JAR cells, though EGFP has been tried to express transiently in JAR cells (12). In the present study, we transfected the gene of EGFP into JAR cells and created a cell line, JAR/EGFP, which stably expressed EGFP. And since EGFP can be applied stably in JAR cells, we fused the genes of EGFP and TCS to the eukaryotic expression vector EGFP-N1, and transfected them into JAR cells by Liposome. The track and distribution of TCS-EGFP in cells as well as the damage to JAR cells were detected by fluorescent microscopy, which provides a platform to research the mechanisms of TCS anti-tumor activity and the development of anti-cancer drugs.

3. MATERIALS AND METHODS

3.1. Materials

The expression vector of pEGFP-N1 was purchased from Clontech Company and the plasmid of wtTCS-pT7 is conserved by our lab. RPMI-1640 medium, Fetal Bovine Serum and LipofectAMINE® Reagent were all from Invitrogen Company. Trypsin, MTT and antibiotic G418 were bought from Sigma Company. The BALB/c nude mice were provided by Cancer Research Institute of Beijing, China.

3.2. Cell culture and liposome-conducted transfection

According to the user manual of LipofectAMINE reagent, 1×10^5 JAR cells were plated in six-pored plates. In 500μl RPMI-1640 medium without antibiotics and serum, 25μl LipofectAMINE reagent was mixed with 2μg plasmid DNA pEGFP-N1 or TCS-pEGFP-N1 and incubated at room temperature for 30 minutes. Then the medium in six-pored plates was removed and the medium containing LipofectAMINE-DNA complex was added into each pores. After 6 hours, the medium was replaced by RPMI-1640 medium with antibiotics and 10% fetal bovine serum.

3.3. Determination of antibiotic G418 concentration for selection

JAR cells were plated in 96-pored plates at 2×10^4/ml and treated with G418 at the concentration of 0, 100, 200, 400 and 800μg/ml respectively. The medium with the same concentration of G418 was refreshed every 3 days. On the 0, 3rd, 6th and 9th day, the MTT assays were performed to detect the survivals of JAR cells, which indicated the modest concentration of G418. Briefly, in each pore, 90μl medium and 10μl MTT (5mg/ml) were added together, and incubated with the cells at 37 ºC for 5 hours. Then the samples were incubated with 100μl acidic SDS (10% m/v SDS containing 0.01 mol/l HCl) at 37 ºC for 12 hours. The OD_{540} was measured on a BioTek Microplate Autoreader to determine the cell viability.

3.4. Creation of JAR/EGFP cell line expressing EGFP stably

The JAR cells transfected with pEGFP-N1 were cultured for 48 hours, and exposed to G418 at the modest concentration. The medium was refreshed every 3 days until the transforming foci could be obviously identified. The foci were pipetted respectively and subcultured in 96-pored plates until each focus contained about 300 to 500 cells. These several hundred cells were digested by trypsin into individuals and each 3~5 cells were distributed into one pore to form monoclones. The cells were continuously exposed to G418. These procedures were performed twice and the obtained cell line, which could grow in G418 medium and express EGFP stably.

3.5. Flow cytometry detection

The cells were digested with 0.25% trypsin and resuspended in PBS (0.01 mol/L, pH 7.4) for a final concentration of 1×10^6 cells/ml. The cell samples were detected in a BD FaCSCalibur flow cytometry. At least 10000 cells' data at 488nm excitation and 510nm emission were collected for each sample.

3.6. Tumor inoculation, separation and subculture

JAR cells were digested into individuals by trypsin and resuspended in RPMI-1640 medium without serum at the final concentration of 2×10^6/ml. Then 200μl cell suspension was hypodermically inoculated into the neck skin of each nude mouse. These mice with tumors were dissected on the 35th day. The tumors were separated in sterile environment and cut into pieces after the fat was gotten rid of. The tumor blocks were digested by trypsin, and then filtered by 350 pores/inch nylon cloth. The eluted cells were centrifuged and subcultured in complete RMPI-1640 medium at 37 ºC.

3.7. Construction of eukaryotic expression vector TCS-pEGFP-N1

PCR was performed to construct the fusion gene. According to the cDNA sequence of TCS (GenBank accession no. **AY669811)**, the PCR primers were designed as followed:

Upstream primer: 5'-GGG AAG CTT ATG GAT GTT AGC TTC CGG-3'; Downstream primer: 5'-GGG CTG CAG TGC CAT ATT GTT TCG ATT-3'. The underlined bases in the upstream primer were the recognition sites of *Hin*dIII followed by a start codon of ATG, and those in the downstream primer were the recognition sites of *Pst*I. The PCR cycle program was set as: denaturation at 94 ºC for 45s, annealing at 54 ºC for 50s and elongation at 72 ºC for 60s. After 35 cycles, the products were electrophoresed in 1% agarose gel. Then the PCR products were digested by *Hin*dIII and *Pst*I and inserted into the vector pEGFP-N1 and sequenced by Sagon Company.

3.8. Fluorescence microscopic observation

After 24 and 48 hours from the transfection, the JAR cells were collected to examine. The cells were fixed

Table 1. The positive rates of JAR/EGFP cells during passages detected by flow cytometry (%)

passages	1	2	3	4	5	6
Clone 8	98.0	81.5	68.8	50.0	28.3	17.5
Clone 23	98.2	95.5	95.0	96.7	97.3	97.9
Clone 41	98.6	90.4	88.9	81.3	75.3	66.5

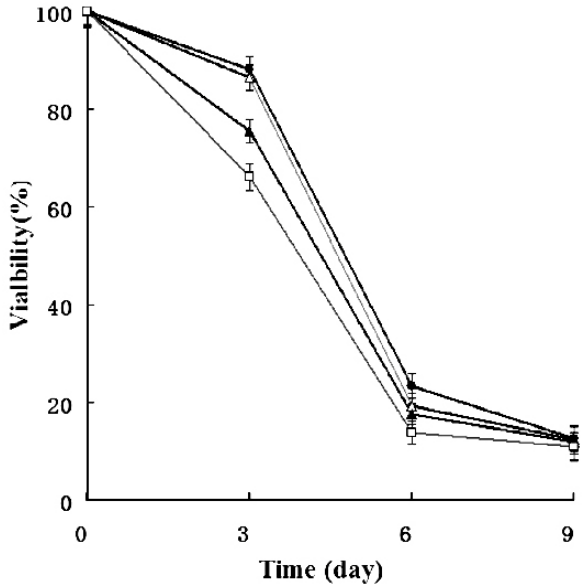

Figure 1. The cytostatic effect of G418 on the survival of JAR cells. The cultured JAR cells were treated with G418 and the concentration of G418 to select JAR/EGFP cells was determined by the cell survivals. Legends: black line with solid round spot, 100μg/ml G418; gray line with hollow triangle, 200μg/ml G418; black line with solid triangle, 400μg/ml G418; gray line with hollow square, 800 μg/ml G418.

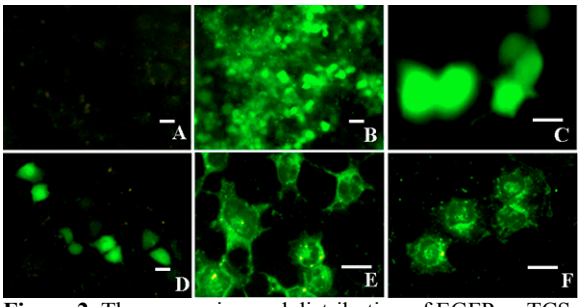

Figure 2. The expression and distribution of EGFP or TCS-EGFP fusion protein in JAR cells visualized by fluorescent microscopy. (bar=10μm) A: Untreated JAR cells as negative control. B & C: JAR cells transfected with pEGFP plasmid. D: Subculture JAR/EGFP cells after inoculation and proliferation in nude mice. E: JAR cells at 24-hour time point after the transfection with TCS-pEGFP-N1 plasmid. F: JAR cells at 48th hour from transfection.

by 4% paraformaldehyde at room temperature for 30 minutes, and then were observed using an OLYMPUS BX51 fluorescence microscopy with the 488nm excitation and 510nm emission.

3.9. Survival determination of JAR cells transfected with TCS-pEGFP-N1

At the time point of 0, 24th and 48th hour from the transfection beginning, the JAR cells transfected with pEGFP-N1 or TCS-pEGFP-N1 were collected to determine the cell survivals by MTT assay as described in section 3.3.

4. RESULTS

4.1. The effect of G418 on the growth of JAR cells

The cytostatic effect of G418 on JAR cells was shown in figure 1. In the group of 800μg/ml G418, the viability of JAR cells decreased about 50% on the fourth day. Accordingly, the concentration of G418 to select positive cells was determined as 800μg/ml, and the lowest concentration of G418 to maintain the selection pressure was used as 400μg/ml.

4.2. Stable expression of EGFP in JAR cells

The fluorescent cell rates of different monoclones during passages were showed in table 1. The positive rates of clone 23 ranged from 95% to 98.2%, but those of clone 8 and 41 decreased quickly. The high and stable positive rates of clone 23 suggested that the EGFP was expressed and inherited stably, and this cell line was named JAR/EGFP. In the fluorescent microscopic observation, the JAR/EGFP cells showed the bright and whole-cell-distributed green fluorescence (figure 2B, C).

4.3. Expression of EGFP in transplanted JAR cells *in vivo*

The tumorigenesis was observed in about 3 weeks after the inoculation and at the end of 5 weeks, the diameters of tumors were approximate 10~15mm. The separated tumor cells from nude mice showed bright and uniform green fluorescence under the microscopy, which had no remarkable different characteristics from the cultured JAR/EGFP cells (figure 2D). This result suggested the EGFP could be expressed stably *in vivo* during the cells proliferated in nude mice.

4.4. Expression of TCS-EGFP in JAR cells and its effects to on JAR cells

The construction of TCS-pEGFP-N1 was illustrated in figure 3. The plasmid was identified by PCR assay and digested by *Hin*dIII and *Pst*I, and then the about 750bp products showed the correct insertion of TCS into pEGFP-N1 (figure 4). The subsequent DNA-sequencing confirmed the construction of TCS-pEGFP-N1.

At the 24th hour later after the plasmid TCS-pEGFP-N1 was transfected into JAR cells, the obvious fluorescence of TCS-EGFP was observed, especially in the cytosol and near the cell membrane, using the fluorescent microscopy (figure 2E). From 24-hour time point to the 48th hour, TCS-EGFP mainly distributed around the nuclear membrane, and the fluorescence in the nucleus strengthened gradually (figure 2F). The fluorescence of EGFP indicated the distribution of expressed TCS from cytosol to nucleus in about 24~48 hours. As described above, TCS was expressed in a mammalian tumor cells for the first time which TCS had highly-sensitive cytotoxicity

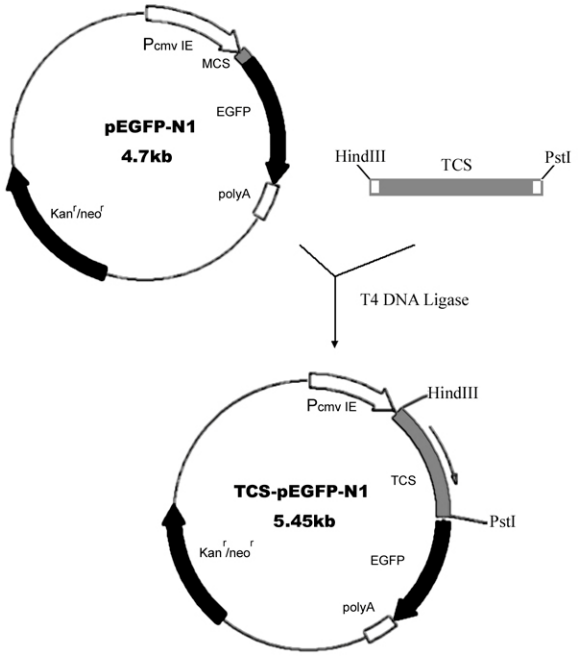

Figure 3. Construction of TCS-pEGFP-N1 plasmid.

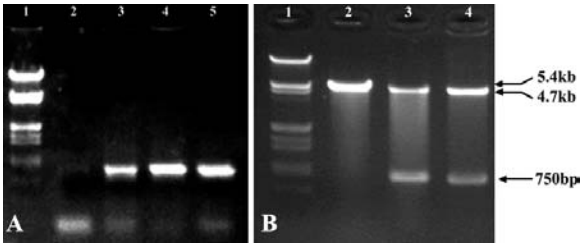

Figure 4. Identification of TCS-pEGFP-N1. Panel A. PCR Determination. Lane 1: lambda DNA/*Hin*dIII+*Eco*RI Molecular Markers; lane 2: negative clone; lane 3-5: positive clones with the ~750bp PCR product. Panel B. Identification by endonuclease digestion. Lane 1: λDNA/*Hin*dIII+*Eco*RI Molecular Markers; lane 2: TCS-pEGFP-N1 plasmid digested by *Pst*I; lane 3-4: TCS-pEGFP-N1 plasmid digested by *Hin*dIII and *Pst*I with the products of ~750bp and 4.7kb fragments

to, and the expression of TCS in JAR cells was not interfered by its inhibition activity to protein synthesis, which indicated that the ribosome-inactivating activity might do not play the most important role in the cytotoxicity of TCS.

Judging from the survivals of transfected JAR cells (figure 5), the living cell amounts in TCS-pEGFP-N1 transfection group decreased remarkably comparing to the controls of pEGFP-N1-transfection group and normal cultured JAR cells, which was identical with the fluorescent cells' death observed in microscopy. The degree of survival decrease□21.2% ± 4.7%□after 48 hours approached to the liposome-transfection efficiency (25%, data not shown), which indicated that the intracellularly-expressed TCS had cytostatic activity to JAR cells.

5. DISCUSSION

The liposome-conducted transfection is an effective method to import heterogenous genes or proteins into host cells. We have successfully transferred FITC-labeled TCS and TCM protein (7), a new member of RIP family, into JAR cells by LipofectAMINE, resulting in the growth inhibition of JAR cells, which showed the high efficiency and feasibility of this transfection. In this study, we transfected the EGFP gene into JAR cells and proved the stable expression of EGFP in JAR cells during passages, which indicated the integration of EGFP gene into the genome of JAR cells. The integrated EGFP genes can be expressed after the JAR/EGFP cells was inoculated into nude mice, which showed EGFP did not interfere the tumor proliferation capacity of JAR cells and this cell line can be used in the animal experiments. In order to apply EGFP as a reporter to track TCS, the vector pEGFP-N1 was chosen. It has been investigated that the N-terminal of TCS was important to its anti-tumor activity (13), therefore the TCS gene was fused to the upstream of EGFP to reduce the possibility to inactivate the cytotoxicity of TCS. Our results built a potential system to investigate the mechanism of TCS cytotoxicity, including the transport and distribution of endogenously expressed TCS in sensitive tumor cells, the morphological changes of tumor cells affected by TCS, and the action pathway of TCS during the cell apoptosis and death.

TCS has been proved to kill several types of tumor cells, but different cell lines showed variant sensitivity to TCS (14), which mechanisms have kept unclear by now. It was generally considered that the anti-tumor activity of RIPs including TCS mainly resulted from that the RIPs inhibited the protein synthesis of tumor cells so as to cause the cell growth inhibition, cell damages and death (14, 15). In fact, the DNase-like activity of TCS may also contribute to its cytotoxicity. Li *et al.* proved that TCS could break the super-coiled DNA into linear and nick types (16), and Bagga *et al.* illustrated the saporin-6, a RIP, could fragment the genomic DNA of cultured U937 cells (17). In our results, the fluorescent TCS protein was observed mainly in the cytosol in 24 hours from the transfection of TCS-pEGFP-N1, and then the fluorescence concentrated around the nuclear membrane and that in the nucleus enhanced in 48 hours. These results showed that TCS-EGFP fusion protein was expressed in cytosol and then some protein transported into nucleus, which may be involved in the cytotoxicity of TCS to JAR cells. So the fragmentation of genomic DNA, as well as the inhibition of protein synthesis and the TCS-induced apoptosis, played important roles on TCS biological functions. After 48 hours, lots of fluorescent cells died, which indicated the endogenously expressed TCS still maintained the cytotoxicity to JAR cells. Furthermore, this fact suggested that the committed step for TCS to kill tumor cells occurred intracellularly, which maybe involved the intrinsic pathway to apoptosis (7, 19).

In conclusion, we provided a fluorescent tumor cell line, which will accelerate the tumor detection, especially in the analyses of Laser Scanning Confocal

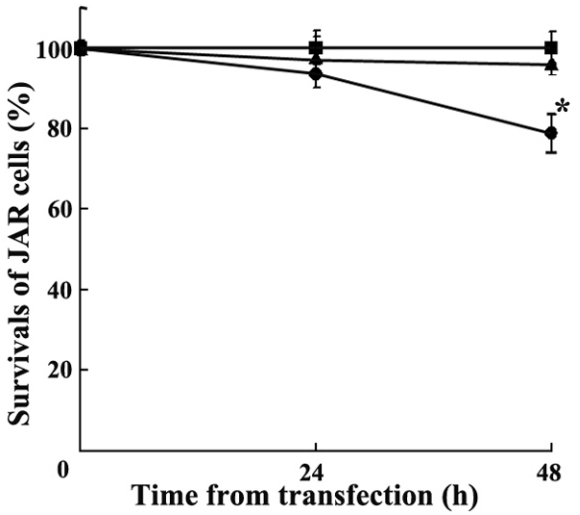

Figure 5. The effects of expressed TCS-EGFP on the survivals of JAR cells. The cell amounts in TCS-pEGFP-N1-transfection group reduced after 24 and 48 hours. The transfection of pEGFP-N1 did not influence the survival of JAR cells remarkably. The survivals of about 80% are due to the relatively low efficiency of liposome-induced transfection. Legends: ■, normal cultured JAR cells as negative control; ▲, JAR cells transfected with pEGFP-N1; ●, JAR cells transfected with TCS-pEGFP-N1. *, $P<0.01$.

Microscopic Tomography. In anti-cancer drug development and cancer therapy trials, the fluorescent reporter can help to track the distribution, proliferation and metastasis of tumor cells (18), analyze the real-time accumulation of anti-tumor drugs *in vivo* and their effects on the target cells.

6. ACKNOWLEDGEMENT

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Abbreviations: TCS: trichosanthin; RIP: ribosome-inactivating protein; (E)GFP: (Enhanced) Green Fluorescence Protein; JAR: Human choriocarcinoma cell line

Key Words: trichosanthin, choriocarcinoma cells, EGFP, liposome-induced transfection

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