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Localization of caspase-3, caspase-8, cytochrome *c*, Hsp27, Hsp72 and LC3 in glioma cells upon quercetin and Temozolomide treatment

Lokalizacja kaspazy-3, kaspazy-8, cytochromu *c*, Hsp27, Hsp72 i LC3 w komórkach glejaka poddanych działaniu kwercetyny i Temozolomidu

SUMMARY

It is well known that the basis of antitumor activity of compounds is the induction of programmed cell death. At the base of this process is the activation and proper localization of marker proteins. Therefore, the aim of our studies was to determine the effect of quercetin and Temozolomide, used separately or in combinations on the localization of proapoptotic proteins such as cytochrome *c*, caspase-3, caspase-8, antiapoptotic Hsp27, Hsp72 and proautophagal LC3 in MOG-GCCM, human *Anaplastic astrocytoma* cell line.

Incubation with quercetin stimulated the translocation of caspase-3 and Hsp72 from the cytoplasm to the nucleus. Temozolomide changed the localization of caspase-3 and Hsp72, causing migration of this protein from the cytoplasm to the nuclei. In the Temozolomide-treated cells caspase-8 was detected mainly in the cytoplasm. Quercetin and Temozolomide administered in combination caused changes in the distribution of tested proteins. Cells treatment with both drugs showed cytoplasmic localization of caspase-8 and nuclear localization of cytochrome *c*. In cells incubated with quercetin and Temozolomide Hsp27 was detected in the whole cell. In the case of cytoplasmic LC3 we did not notice any significant changes in distribution.

Key words: *Anaplastic astrocytoma*, caspase-3, caspase-8, cytochrome *c*, Hsp27, Hsp72, LC3, quercetin, Temozolomide

STRESZCZENIE

Powszechnie wiadomo, że podstawą antynowotworowego działania związków jest indukcja zaprogramowanej śmierci. U podstawy tego procesu leży aktywacja oraz odpowiednia lokalizacja białek markerowych. W związku z tym celem naszych badań było określenie wpływu kwercetyny oraz Temozolomidu, stosowanych razem lub osobno, na lokalizację proapoptotycznych białek, jak cytochrom *c*, kaspaza-3, kaspaza-8, antyapoptotycznych Hsp27, Hsp72 oraz proautofagalego białka LC3 w komórkach *Anaplastic astrocytoma*. 24-godzinna inkubacja z kwercetyną spowodowała migrację kaspazy-3 oraz Hsp72 z cytoplazmy do jądra. Temozolomid stymulował translokację kaspazy-3 i Hsp72 z cytoplazmy do jądra. Ponadto komórki MOGGCCM traktowane Temozolomidem wykazywały cytoplazmatyczną lokalizację kaspazy-8. Komórki traktowane kwercetyną i Temozolomidem spowodowały translokację kaspazy-8 do cytoplazmy i cytochromu *c* do jądra. W komórkach MOGGCCM traktowanych kombinacjami leków zanotowano obecność Hsp27 na terenie całej komórki. W przypadku cytoplazmatycznego LC3 nie stwierdzono znaczących zmian w lokalizacji.

List of abbreviations: AA – *Anaplastic astrocytoma*; Apaf-1 – Apoptosis protease activating factor-1; DMEM – Dulbecco's Modified Eagle Medium; FBS – Fetal Bovine Serum; FITC – Fluorescein Isothio**c**yanate; GBM – *Glioblastoma multiforme*; MGMT – O6-methylguanine-DNA methyltransferase; MOGGCCM – Human brain astrocytoma cell line; MTIC – 5-(3-methyltriazen-1-yl) imidazole-4-carboxamide; PI3K – Phosphoinositide-3 kinase; PKC – Protein Kinase C; Ras – a family of proteins involved in cellular signal transduction; TMZ – Temozolomide.

INTRODUCTION

Quercetin (3,3',4',5–7-pentahydroxyflavone) is one of the most popular and widespread flavonoid in the plant kingdom. This compound is a component of most edible fruits and vegetables like apples, grapes, lemon, tomato, kale and onion. While people consume 30 mg of quercetin in the daily diet, only 30–50% of ingested flavonoid is absorbed and the rest passes through the gastrointestinal tract. Many studies have shown that quercetin actively participates in many different processes. It down regulates mutant p53 protein, blocks the cell cycle in the late G1 phase, blocks estrogen II binding sites, inhibits the expression of proteins (Hsp and Ras) and the activity of several enzymes (PI3K, PKC, tyrosine kinase, xantine oxidase) (Dajas et al. 2003, Jakubowicz-Gil et al. 2008 (a), Jakubowicz-Gil et al. 2008 (b), Lamson, Brignall 2000). Recent investigations demonstrated that quercetin displayed antitumor activity by triggering apoptosis in numerous types of cancer such as breast cancer, lung cancer, ovarian cancer, hepatoma cancer. It was correlated with the activation of proapoptotic proteins caspase-3, caspase-8 and cytochrome *c*, and their changes in localization. It was also accompanied by the inhibition of Hsp expression (Lamson, Brignall 2000).

Furthermore, it was suggested that this flavonoid enhanced the therapeutic effectiveness of doxorubicin, cisplatin and cyclophosphamide – chemotherapeutic agents used in cancer treatment. Our earlier experiments revealed that quercetin acts synergistically with Temozolomide in programmed cell death induction in gliomas (Du et al. 2010, Jakubowicz-Gil et al. 2010).

Temozolomide (TMZ) is an alkylating cytostatic used in the treatment of primary or recurrent high-grade gliomas, including anaplastic astrocytoma and glioblastoma multiforme (Kanzawa et al. 2004, Koukourakis et al. 2009). The compound is a prodrug hydrolysing spontaneously at physiological pH into the active form MTIC (5-(3-methyltrazen-1-yl) imidazole-4-carboxamide) (Gliński et al. 2006). It is responsible for O6-guanine methylation, which creates abnormal pair with tymine. Invalid couple is recognized as an error, which stops the DNA synthesis, arrests cell cycle in G2/M and induces autophagy in consequence. It was found that the survival period of patients with GBM treated with Temozolomide was longer (Friedman et al. 2000, Klonowicz et al. 2007).

It is well known that protein localization may be a useful marker of cell death. Thus the aim of the present study was to investigate the effect of TMZ and quercetin administered alone or in combinations to the localization of proapoptotic caspase-3, caspase-8, cytochrome *c*, antiapoptotic Hsp27, Hsp72 and proautophagal LC3 in human glioma cell line MOGGCCM.

MATERIAL AND METHODS

Cells and culture conditions

The human brain astrocytoma cells (MOGGCCM) were grown in 1:1 mixture of DMEM and Nutrient mixture F-12 Ham (Ham's F-12) (Sigma) supplemented with 10% FBS (Life Technologies, Karlsruhe, Germany), penicillin (100 μg/ml) (Sigma) and streptomycin (100 μg/ml) (Sigma). The cultures were kept at (37°C) in humidified atmosphere of 95% air and 5% CO_2.

Drug treatment

Quercetin (Sigma) at the final concentrations 5, 15, 30, 45 μM and Temozolomide (Shering-Plough) at the final concentrations 5, 10, 25, 50, 100 μM were used in the experiments. Drugs were dissolved in dimethyl sulfoxide (DMSO, Sigma). The final concentration of DMSO in the culture medium did not exceed 0.01%, which as indicated in preliminary experiments, did not influence cell viability and the expression of studied proteins. Three variants of drug treatment were performed. In the first one MOGGCCM cells were incubated only with quercetin or Temozolomide for 6, 12, 24, 48 and 72 h. In another, quercetin and Temozolomide were added to the culture medium at the same time and incubated for 24 h. In the last variant:

- the cells were preincubated with quercetin for 6 h followed by Temozolomide treatment for the next 18 h;
- the cells were preincubated with Temozolomide for 6 h followed by quercetin addition for the next 18 h;

As control, cells were incubated with 0.01% of DMSO.

Indirect immunofluorescence

After quercetin and/or Temozolomide treatment the cells were washed three times with PBS and fixed in 3.7% paraformaldehyde (Sigma) in PBS for 10 min. After extensive washing with PBS the cells were treated with 0.2% Triton X-100 (Sigma) for 7 min and then washed three times with PBS, all at the room temperature. Later a blocking step of 30 min long incubation in 5% low fat milk at the room temperature was included. Then the cells were incubated with: mouse monoclonal antibody anti-Hsp72 (SPA 810, StressGen) diluted 1:200, anti-Hsp27 (SPA 800, StressGen) diluted 1:100, anti-caspase-8 (Sigma) diluted 1:30, and rabbit antibodies anti-caspase-3 active form (Sigma) diluted 1:50, anti-LC3 (Sigma) diluted 1:30 and sheep anti-cytochrome *c* antibody (Sigma) diluted 1:300. The primary antibodies were detected with FITC (Fluorescein Isothiocyanate)-conjugated anti-mouse, anti-rabbit or anti-sheep secondary antibodies (Sigma). The localization of proteins in cells was analysed using PASCAL5 scanning head (Zeiss). Pictures were registered within fluorescence channel (λ = 488 nm). Three independent experiments were performed. Over 100 cells were analysed in each experimental variant.

RESULTS

The effect of quercetin on proteins' localization

Human astrocytoma cell line MOGGCCM was incubated with quercetin for 12, 24, 48 and 72 h. Indirect immunofluorescence showed that incubation with

quercetin at all concentrations and all time incubations did not change the distribution of caspase-8 (Fig. 1a), cytochrome *c* (Fig. 1b), Hsp27 (Fig. 1c) and LC3 (Fig. 1d) within cells. Caspase-8 was located in the nucleus, while Hsp27, cytochrome *c* and LC3 were found in the cytoplasm. Changes in the localization were observed in the case of caspase-3 and Hsp72. Distribution of caspase-3 (Fig. 1e-h) was concentration dependent. In control cells the protein was located mainly in the cytoplasm and slightly in the nuclei (Fig. 1e). Starting from 5 μM no changes in the localization of this protein were noticed in comparison to the control. Treatment with higher concentration (15 μM) caused gradual migration of caspase-3 from the cytoplasm to the nuclei (Fig. 1f). At the concentration 30 μM greater amount of protein was clearly visible in the nuclei (Fig. 1g). In the case of Hsp72 (Fig. 1h), in control cells the protein was located in the cytoplasm. Incubation with all quercetin concentration, at all incubation time caused Hsp72 migration toward the nuclei (Fig. 1i).

The effect of Temozolomide on proteins' localization

Incubation of MOGGCCM cells with Temozolomide for 12, 24, 48 and 72 h did not change the distribution of cytochrome *c*, LC3 and Hsp27 within cells (data not presented) and these proteins were present in the cytoplasm. Temozolomide changed the localization of caspases. In the case of caspase-3 incubation with 5 μM of drug stimulated the protein translocation from cytoplasm to the nuclei (Fig. 2a). After incubation with 50 μM the majority of caspase-3 was detected in the nuclei (Fig. 2b). Incubation with 50 μM of Temozolomide resulted in translocation of the protein caspase-8 from the nuclei to the cytoplasm (Fig. 2c), while lower concentration had no significant effect on protein localization in comparison to control cells. Localization of Hsp72 was concentration dependent. Cells treated with minimal (5 μM) TMZ's concentration showed nuclear location of Hsp72 (Fig. 2d). Incubation with 100 μM caused gradual return of Hsp72 from nuclei to the cytoplasm (Fig. 2e).

The effect of combination of quercetin and Temozolomide on proteins' localization

To examine the effect of quercetin and Temozolomide on studied proteins' localization in MOGGCCM cell line, astrocytoma cells were treated with both drugs at the same time for 24 h. Additionally preincubation variants with quercetin or TMZ treatment were included (detailed description in MATERIAL AND METHODS). Preincubation of MOGGCCM cells with quercetin did not change the primeval distribution of caspase-3, Hsp27, Hsp72 and LC3 and proteins were detected in the cytoplasm (data not presented). Changes occurred only in the case of other proapoptotic proteins. When the quercetin at the concentration 30 μM was co-administered with 100 μM of TMZ, small quantities of cytochrome *c* were

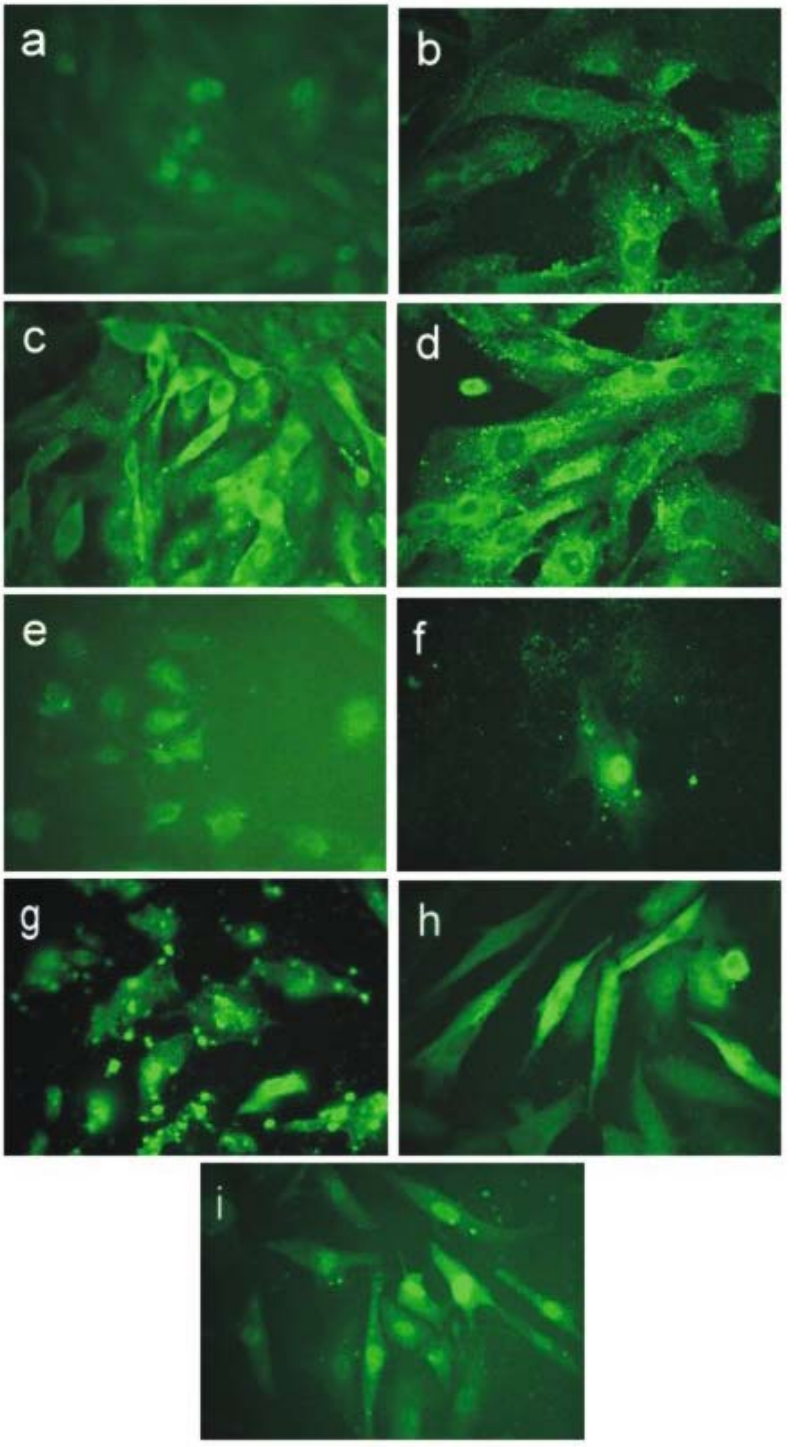

Fig. 1. Localization of caspase-3, caspase-8, cytochrome *c*, Hsp27, Hsp72, LC3 in astrocytoma cells incubated with quercetin for 24 h, revealed by indirect immunofluorescence; (a) caspase-8; (b) cytochrome *c*; (c) Hsp27; (d) LC3; (e) caspase-3 in control (non-treated) cells; (f) caspase-3 after 15 μM and (g) 30 μM of quercetin; (h) Hsp72 in control sample; (i) Hsp72 after incubation with 30 μM of quercetin

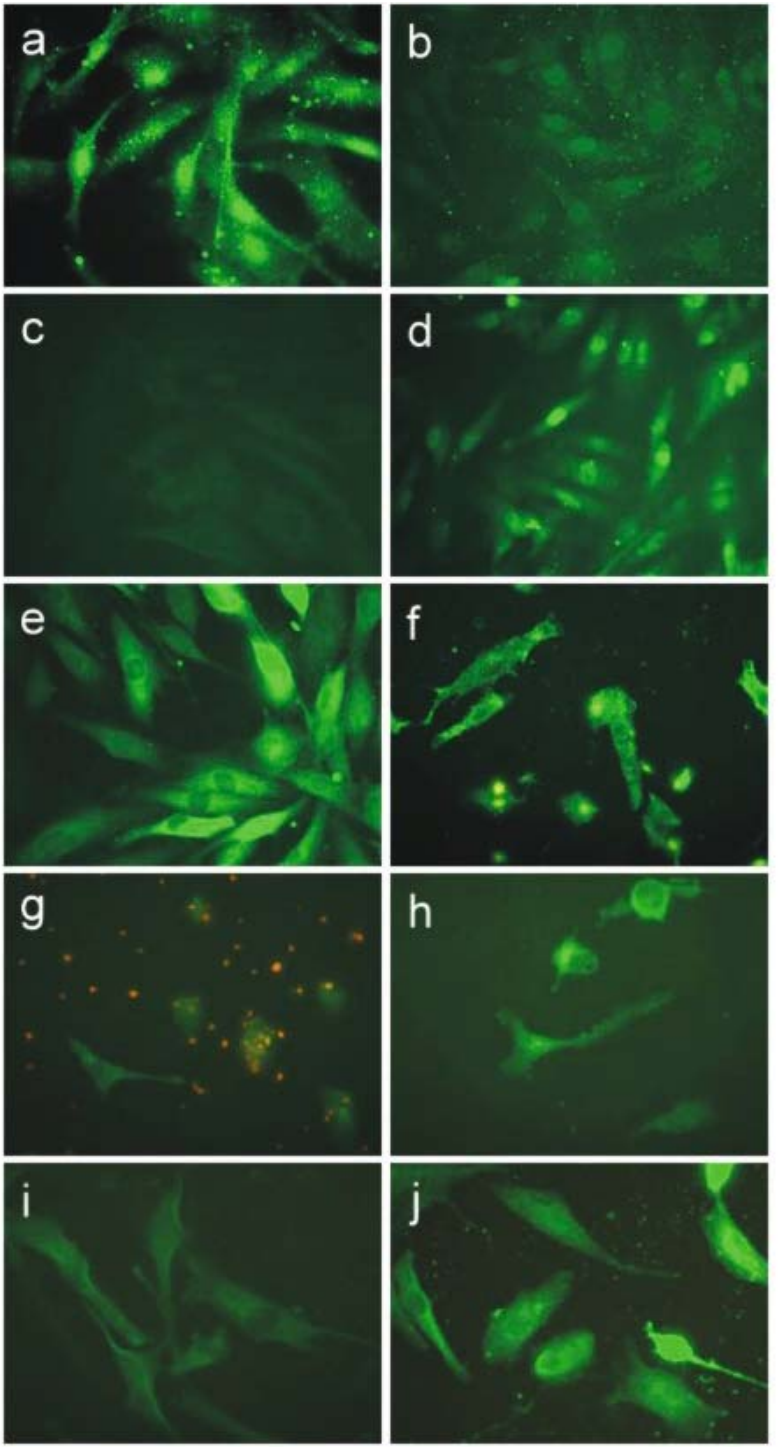

Fig. 2. Distribution of caspase-3, caspase-8, cytochrome *c*, Hsp27, Hsp72, LC3 in astrocytoma cells revealed by indirect immunofluorescence; (a) caspase-3 after incubation with TMZ (5 μM) for 24 h; (b) caspase-3 after incubation with TMZ (50 μM) for 24 h; (c) caspase-8 after incubation with TMZ (50 μM) for 24 h; d) Hsp72 after incubation with TMZ (5 μM) for 24 h; (e) Hsp72 after incubation with TMZ (100 μM) for 24 h; (f) cytochrome *c* after preincubation with quercetin (30 μM) followed by TMZ (100 μM) treatment; (g) caspase-8 after preincubation with quercetin (30 μM) followed by TMZ (100 μM) treatment; (h) caspase-8 after preincubation with TMZ (100 μM) followed by quercetin (30 μM) treatment; (i) caspase-8 treated with TMZ (100 μM) and quercetin (30 μM) given simultaneously; (j) Hsp27 after TMZ (100 μM) and quercetin (15 μM) given simultaneously

detected in the nuclei (Fig. 2f). Drug combination described above stimulated translocation of caspase-8 from the nuclei to the cytoplasm (Fig. 2g).

Preincubation with Temozolomide did not change the distribution of caspase-3, cytochrome *c*, Hsp27, Hsp72 and LC3 in cell in relation to control sample (data not presented). Preincubation with the highest concentration of drug (100 μM) co-administered with 30 μM of quercetin caused migration of caspase-8 from the nuclei to the cytoplasm (Fig. 2h).

Administration of both drugs at the same time did not change the localization of caspase-3, cytochrome *c*, Hsp72 and LC3 and these proteins were localized in the cytoplasm (data not presented). Application of TMZ at the highest concentration (100 μM) with the quercetin at the concentration 15 μM and 30 μM, resulted in migration of caspase-8 from nuclei to the cytoplasm (Fig. 2i). MOGGCCM cells treated with Temozolomide at the highest concentration co-administered with flavonoid at the concentration 15 μM showed Hsp27 in the whole cell (Fig. 2j)

DISCUSSION

Anaplastic astrocytoma is one of the most malignant tumors. The median survival time is about 3–5 years after applying the standard treatment including resection with postoperative radiotherapy. Elimination of *Anaplastic astrocytoma* cells represents a real challenge. At the molecular level tumor cells activate the mechanism blocking apoptosis and autophagy, which contributes to a resistance to chemotherapy. That is why scientists are still looking for ways of overcoming it (Jiang et al. 2009, Kondo, Kondo 2006, Tanida et al. 2004).

One of the most common proteins overexpressed in tumor cells are heat shock proteins Hsp27 and Hsp72. They belong to the large family of Hsp (Heat shock proteins), well known molecular chaperones, protecting cell against stressors and preventing apoptosis. Under physiological conditions these proteins are distributed throughout the cytoplasm where they are involved in the regulation of protein synthesis and of proper protein folding (Kaźmierczuk, Kiliańska 2009). They are also engaged in the degradation of misfolded or denaturated proteins. Hsp72 protein is also known as a molecular marker of stress. Under stress conditions Hsp72 translocates to the nucleus, where it protects nuclear structures and transcription processes. This phenomenon is very unfavourable in the case of tumors, as it increases the resistance of cancer cells to chemotherapy treatment (Garrido et al. 2003, Kaźmierczuk, Kiliańska 2009). Thus the blocking of nuclear localization of Hsps would be beneficial for treatment.

In our study incubation of MOGGCCM cells with quercetin stimulated Hsp72 migration to the nucleus. What was interesting, incubation only with Temozolomide, as well as in combination with the flavonoid had no significant effect on proteins distribution. This result may suggest that Temozlomide inhibits

the translocation of Hsp72 protein to the nucleus stimulated by quercetin, which in consequence may increase cells resistance to apoptosis induction. Quercetin and Temozolomide applied separately or in combinations had no effect on Hsp27 distribution in MOGGCCM cells. Nuclear localization was observed only after simultaneous treatment with Temozlomide at the concentration 100 μM and quercetin at the concentration 15 μM, which may suggest the toxic combination.

It is well known that apoptosis is a type of programmed cell death, based on the activation of proteins called caspases. The process may be initiated in two ways: "extrinsic", with the activation of membrane death receptor and caspase-8, and "intrinsic", associated with the release of cytochrome *c* from mitochondrium and subsequent activation of caspase-9. Both types lead to caspase-3 activation (Baliga, Kumar 2003).

It was observed that during apoptosis caspase-8 translocates to the cytoplasm, which is a signal of cell damage (Baliga, Kumar 2003). Such changes in the localization of the protein were observed after simultaneous treatment of MOGGCCM with Temozolomide (100 μM) and quercetin (15 μM and 30 μM) or in the case of cells preincubated with 100 μM of Temozolomide. Such results may suggest that Temozolomide induces apoptosis by externistic pathway, but this process may be blocked by preincubation with quercetin, which blocks caspase-8 in nuclei.

Caspase-3, an effector caspase in both apoptosis pathways, was observed on relatively small degree in our cell line. Quercetin (15 μM and 30 μM) stimulated the translocation of this protein to the nucleus, which may be the effect of proapoptotic activity of quercetin (Lamson, Brignall 2000). Similar results were achieved in the case of incubation with 5 μM and 50 μM of Temozolomide. Interestingly, incubations with both drugs had no effect on the localization of caspase-3.

Cytochrome *c* is a protein localized in the space between the inner and outer mitochondrial membranes. During apoptosis it is released into the cytoplasm where it binds with adapter proteins, which initiates the activation of caspases (Baliga, Kumar 2003, Crow et al. 2004). This process is also accompanied by the gradual accumulation of cytochrome *c* in the nucleus, where the protein is involved in the remodelling of chromatine (Nur-E-Kamal et al. 2004). Our experiments demonstrated the nuclear localization of cytochrome *c* after preincubation with 30 μM of quercetin followed by the treatment with 100 μM of Temozolomide.

It is well known that autophagy is an alternative type of programmed cell death in gliomas. At the molecular level this process is correlated with the conversion LC3 protein into an active form of LC3-II, which binds to the insulating membrane of autophagosome (Jiang et al. 2009, Kondo, Kondo 2006, Kuma et al. 2007, Mizushima, Yoshimori 2007, Tanida et al. 2004). In our experiments quer-

cetin and Temozolomide applied alone or in combinations had no effect on LC3 distribution and the protein was located in the cytoplasm.

CONCLUSIONS

Our results indicate that the effect of quercetin and Temozolomide on the localization of proapoptotic proteins such as cytochrome *c*, caspase-3, caspase-8, antiapoptotic Hsp27, Hsp72 and proautophagal LC3 in MOGGCCM cells depended on the type of drug. Quercetin changed the distribution of caspase-3 and Hsp72, while incubation with TMZ changed the localization of both caspases and Hsp72. Temozolomide in combination with quercetin redistributed caspase-8, cytochrome *c* and Hsp27. Such changes may have an impact on the increased sensitivity of tumor cells to apoptosis induction, but the problem needs more detailed experiments.

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