Liver cancers are the second leading cause of cancer death in the world (4). Hepatocellular carcinoma (HCC) is a major subtype among primary liver cancers occurring in humans, dogs and cats. The prognosis of advanced HCC is poor. With its resistance to chemotherapy and rapid progression the tendency is to look for new effective methods of therapy to combat this cancer using the immune system of the host (11). In the tumour microenvironment, innate immune cells, represented by macrophages, can be polarized into classically activated M1 and alternatively activated macrophages M2 (17). The M1 phenotype is pro-inflammatory and is characterized by the release of inflammatory cytokines, reactive nitrogen intermediates (RNI), reactive oxygen species (ROS) and microbiocidal/tumoridical activity. Using an in vitro approach, some authors have demonstrated that a short priming of monocytes with barley-derived β-glucan (BBG) induces the trained immune cells with an enhanced inflammatory status (13, 15). In contrast, M2 macrophages have an immunosuppressive and tumor-promoting phenotype.

The peroxisome-proliferator-activated receptor gamma (PPARγ) is a nuclear transcription factor member of the nuclear hormone receptor superfamily which plays a significant role in several diseases and pathological conditions, such as carcinogenesis (10, 14). However, the current reports describe contradictory action of PPARγ in cancer. While some studies have implicated PPARγ in the promotion and develop-
opment of cancer, others, in contrast, have presented evidence for a protective role of this receptor against carcinogenesis (19). In the case of human HCC the same conflicting results have been noticed. Koga et al. found that there were no significant differences in PPARγ expression between HCC tissues and adjacent non-tumorous liver tissue (6). On the other hand, Lin et al. found an increased mRNA expression of PPARγ in HCC compared with that in normal liver (9).

Taking the above into account, we sought to analyse the proliferative activity and PPARγ expression in neoplastic and non-neoplastic rat hepatocytes exposed to immunologically trained macrophages M1 (Mf-M1).

Material and methods

Ten-week-old female Wistar rats weighing 200-250 g were used in this study. The animals were kept in a temperature- and humidity-controlled room with a 12 h light-dark cycle.

After a 1-week period of acclimatization, the rats were divided into two groups: I – control group (n = 5) and II – neoplastic group (n = 5). To induce HCC in the neoplastic group, genotoxic diethylnitrosamine (DEN) (Sigma Aldrich, Poznań, Poland) was administered after a partial hepatectomy (PH), (5,17). The partial hepatectomy was performed by excision of left lateral and right lobes (2/3 partial heptectomy (PH), (5,17). The partial hepatectomy was performed by excision of left lateral and right lobes (2/3 partial heptectomy) according to the Higgins and Anderson method. Seven days after PH, 0.005% DEN was administered in drinking water for 6 weeks. All procedures were approved by the local ethical committee (decision number 81/2015).

After 6 weeks of DEN administration, hepatocytes were isolated from both groups of animals as described previously, with minor modifications (16). After laparotomy, the liver was perfused in situ through the portal vein with a Krebs-Ringer buffer made up of three parts as follows: i) containing Disodium ethylenediaminetetraacetate dihydrate (EGTA) (Sigma Aldrich, Poznań, Poland); ii) without Ca²⁺ and chelating agent; and iii) with type IV collagenase (Sigma Aldrich, Poznań, Poland). The viability of isolated cells, estimated by the Trypan-blue exclusion method, ranged between 75 and 85%. Finally, adhered control and DEN-obtained hepatocytes were cultured in DMEM/HAMS-12 (v/v) medium supplemented with 10% calf serum (Biomed Lublin, Poland) and a mixture of antibiotics.

Isolation of rat blood-derived monocytes. Blood was collected from each animal during the experimental procedure but before liver perfusion. The mononuclear cells were isolated using Lymphoprep density-gradient centrifugation. After being counted (viability > 80%), cells were plated into wells of a 96-well plate at a density of 1.0 × 10⁶ cells/ml and cultured at 37°C with 5% CO₂ for 24 h in Dulbecco’s modified Eagle’s medium (Sigma Aldrich, Poznań, Poland) with 10% of calf serum (Biomed Lublin, Poland) (17).

The adhered cells were cultured for the next 48 h to allow monocytes to mature into functional macrophages. After differentiation, cells were treated with BBG (Sigma-Aldrich, Poznan, Poland) at a concentration of 10 µg/ml or with phosphate buffered saline (PBS; Biomed, Lublin, Poland) in equal volumes and incubated for 24 h. All func-

![Fig. 1. Scheme of the experimental procedure. Using Quasi-Vivo Cell Culture Flow Systems, non-stimulated and stimulated macrophages were co-cultured with control and neoplastic hepatocytes during 24 h](image)

**Explanation:** A phase-contrast micrograph shows the isolated and cultured Mf, Mf-M1 and control and neoplastic hepatocytes. Magnifications are the original microscope magnification (200 ×)

**Materials and Methods:**

- **Animals:** Ten-week-old female Wistar rats weighing 200-250 g were used in the study. The animals were kept in a temperature- and humidity-controlled room with a 12 h light-dark cycle.
- **Hepatocytes Isolation:** After a 1-week period of acclimatization, the rats were divided into two groups: I – control group (n = 5) and II – neoplastic group (n = 5). To induce HCC, DEN was administered after partial hepatectomy (PH), (5,17). The partial hepatectomy was performed by excision of left lateral and right lobes (2/3 partial heptectomy) according to the Higgins and Anderson method. Seven days after PH, 0.005% DEN was administered in drinking water for 6 weeks. All procedures were approved by the local ethical committee (decision number 81/2015).
- **Macrophages Culture:** After 6 weeks of DEN administration, the hepatocytes were isolated from both groups of animals as described previously, with minor modifications (16). After laparotomy, the liver was perfused in situ through the portal vein with a Krebs-Ringer buffer made up of three parts as follows: i) containing Disodium ethylenediaminetetraacetate dihydrate (EGTA) (Sigma Aldrich, Poznań, Poland); ii) without Ca²⁺ and chelating agent; and iii) with type IV collagenase (Sigma Aldrich, Poznań, Poland). The viability of isolated cells, estimated by the Trypan-blue exclusion method, ranged between 75 and 85%. Finally, adhered control and DEN-obtained hepatocytes were cultured in DMEM/HAMS-12 (v/v) medium supplemented with 10% calf serum (Biomed Lublin, Poland) and a mixture of antibiotics.

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**Adherent Hepatocytes and Mf** were put into the simple modular culture chambers with the dynamic flow of culture medium controlled by a peristaltic pump (Quasi-Vivo System, Kirkstall Ltd., UK) (Fig. 1). This dynamic 3D culture was maintained during 24 hours. Next, hepatocytes were taken for analysis after 72 h and then after the 1st, 2nd and 3rd weeks of incubation.

**MTT analysis-assessment of cell proliferation was based on the reduction of tetrazolium salt into blue formazan by the mitochondrial dehydrogenase of viable cells** (7, 12). Cultures were pulsed with 15 µl of the MTT solution (3-[4,5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazolium bromide) for 3 hours at 37°C and solubilized to dissolve the dark blue crystals overnight. Microplate reader (Alab Plate Reader ELISA) measured the optical density (OD) of the formed blue formazan at the wavelength of 600 nm. The results were expressed as a proliferation index (PI).

The hepatocyte protein expression of PPARγ was measured in cell lysates using a rat PPARγ Assay ELISA Kit (Biotechist, Beijing, China) according to the manufacturer’s instructions. Measurement was done at 450 nm against Reagent Blank (Alab Plate Reader ELISA).

**Statistical analysis.** The obtained values were compared using Microsoft Excel and STATISTICA.PL 13.1 software (StatSoft Poland). Results were expressed as the mean ± SD, and the data obtained were evaluated by a one-way analysis of variance (ANOVA) and Student’s t-test as appropriate. The level of significance was set at P < 0.05.
Results and discussion

Unstimulated rat macrophages were rounded, whereas under the influence of BBG, cells generated long filopodia, especially in cells from DEN-treated rats (Fig. 1).

As shown in Figure 2 the proliferation index of control hepatocytes ranged between 0.41 ± 0.04-0.43 ± 0.03 at 72 h and after the 3rd week of incubation, respectively. Exposure of control hepatocytes to non-stimulated macrophages resulted in marked increase of IP after the 1st, the 2nd and the 3rd week of incubation. In turn, when hepatocytes were influenced by trained macrophages M1, their proliferation was maintained at the control stage. DEN-obtained hepatocytes, not influenced by macrophages, exhibit enhanced proliferation (Fig. 3). Exposure of these neoplastic cells to non-stimulated macrophages resulted in augmentation of IP, especially after the 1st and the 3rd weeks of the experiment. Markedly (P ≤ 0.05) inhibited cell proliferation was observed in hepatocytes co-cultured with Mf-M1. At this stage of our study, the value of IP did not exceed 0.45 ± 0.05.

Expression of PPARγ in control hepatocytes was maintained at a comparable level regardless of macrophages effect (Fig. 4). In co-culture of neoplastic hepatocytes with non-stimulated Mf, we did not observe any significant response in the PPARγ concentration. Exposure of DEN-obtained hepatocytes to polarized Mf M1 caused notable (p ≤ 0.05) increase of PPARγ in each point of experimental time (Fig 5). Maximum expression of PPARγ was noticed at the 72h of culture and reached the value of 1.22 ± 0.09 ng/ml. Concomitantly with time of incubation, the expression of PPARγ markedly decreased and reached the value of 0.62 ± 0.09 ng/ml. Statistical analysis revealed a high negative relationship (r = –0.93) between the medium concentration of PPARγ and the proliferative activity of the DEN-obtained hepatocytes (Fig. 6).

The reports about the expression of PPARγ in HCC tissue show conflicting results (3, 4). Some authors noticed that activation of PPARγ promotes HCC, while another pointed out the inhibitory effect of PPARγ on growth and viability of HCC cells (18). In this study, we have presented the evidence for the expression of PPARγ in healthy and neoplastic hepatocytes influenced by macrophages in vitro.

The obtained results show that there were no significant differences in PPARγ expression between control and neoplastic cells incubated in pure culture medium. It should be stressed that this slight expression of PPARγ in healthy cells was maintained independently from their exposure to non-activated Mf and activated Mf-M1. Thus, our results confirmed the scope of observations that in normal liver, PPARγ is known to be expressed at a low level (2).

We observed different responses in neoplastic cells. When the neoplastic cells were co-cultured with non-activated Mf, an insignificant increase of PPARγ concentration was found. Under the influence of Mf-M1, a significant (p ≤ 0.05) elevation of PPARγ concentration was demonstrated. As was described previously, the classically activated M1 macrophages up-regulate the expression of the pro-inflammatory cytokines, including TNFα, IL-6 and interferon-γ, and in this way may activate the hepatic PPARγ (1, 8). According to Yu et al., the overexpression of PPARγ inhibits HCC cell growth in a time dependent manner (20). Cell cycle analysis revealed that PPARγ overexpressing cells were often arrested in the G2/M phase, in contrast...
to the G0/G1 arrest by troglitazone as reported previously. On the other hand, as was noticed by Nojima et al., PPARγ activation is reported to inhibit the ubiquitination of p27(kipl) by Skp2, decrease the expression of cyclin D1, and induce cell cycle arrest by inhibiting S phase entry in HCC cells in vitro (11). The effects of the natural PPARγ agonist, 15-deoxy-Δ12,14-prostaglandin J2, and the synthetic agonist, thiazolidine-diones, have been tested in many human HCC cell cultures (2). Overall, the cell growth was inhibited at variable concentrations of 15-deoxy-Δ12,14-prostaglandin J2 and thiazolidine-diones. In turn, Hsu et al. found that natural and synthetic PPARγ agonists exhibit not only a growth inhibitory effect in HCC, but also induce apoptosis (4). In our experiment, the neoplastic hepatocytes cultured in pure medium proliferated more actively than control cells. Under the influence of non-stimulated Mf proliferative activity of DEN-obtained hepatocytes did not decrease. After the 1st and the 3rd week of incubation we even observed an increase of proliferation of these cells. When the neoplastic hepatocytes were exposed to Mf-M1, a significant inhibition of their proliferative activity was detected. It should be stressed that this limited proliferation was correlated with the intensified expression of PPARγ.

Taking into account our results, we conclude that the immunologically trained macrophages M1 are capable of the activation of PPARγ in rat neoplastic hepatocytes derived from experimentally induced HCC. In turn, the intensified expression of PPARγ may inhibit the proliferation of neoplastic cells in vitro. However, the precise mechanism and the critical downstream effectors of PPARγ in the relation to its therapeutic efficacy in HCC need the further research.

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