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Extended Abstract

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Biotechnology of tumor growth restriction: M3 macrophage switch phenotype and antigen-reprogrammed lymphocytes

Malyshev Igor

Moscow State University of Medicine

E-mail: iymalyshev1@gmail.com

Many tumors produce anti-inflammatory cytokines, which reprogram antitumor M1 macrophages to protumor M2 macrophages via activation of transcription factors, STAT3, STAT6, and SMAD3. Earlier we showed that M1 macrophages with inhibited STAT3, STAT6, and SMAD3 (M3 phenotype) responded to the action of protumor, anti-inflammatory cytokines by increasing production of antitumor, proinflammatory cytokines and thus, preserved their antitumor properties. In vivo, the tumor also disorders the antigen presentation by macrophages and prevents formation of antigen-specific T and Th1 lymphocytes with strong antitumor properties. We hypothesized that presentation of tumor antigens to lymphocytes by M3 macrophages in vitro, in absence of tumor cells, could result in an effective antitumor programing of the lymphocytes. It can be expected that a composite pool of M3 macrophages and in vitro antigen-reprogramed lymphocytes would effectively restrict tumor growth. We showed that the antitumor effect of M3 macrophages depended on timing of their administration following the onset of solid tumor development. In early administration, M3 macrophages partially restricted the tumor development. In late administration, M3 macrophages restricted the tumor growth but to a significantly less extent. Adding antigenreprogramed lymphocytes to M3 macrophages resulted in complete inhibition of tumor growth both in vitro and in vivo, both in early and late administration. The fact that M3 macrophages and antigen-reprogramed lymphocytes completely suppressed tumor growth makes it very promising to develop a clinical biotechnology for reducing the tumor growth by prior in vitro antitumor programing of the immune response. Macrophages play the key role in immune disorders during carcinogenesis. A concept put forward by Mills et al. and supported by other researchers, implies that, depending on the microenvironment, macrophages reprogram themselves into either an M1/kill phenotype or an M2/repair phenotype. The M1 phenotype can destroy tumors due to production of nitric oxide, pro-inflammatory cytokines, activation of natural killers, and presentation of tumor antigens to lymphocytes. Pro-inflammatory cytokines acting on macrophages shift their phenotype even more towards the M1, which produces more pro-inflammatory cytokines. Thus, the pro-inflammatory mechanism involves positive feedback. However, many tumors produce anti-inflammatory cytokines, such as TGF-β, IL-10, and IL-13, which reprogram the anti-tumor M1 phenotype into the pro-tumor M2 phenotype. The M2 phenotype produces large quantities of anti-inflammatory cytokines and has a low capability for presentation of tumor antigens. Anti-inflammatory cytokines acting on macrophages shift their phenotype even more towards the M2, which produces more anti-inflammatory cytokines. Thus, the anti-inflammatory mechanism involves positive feedback. The M2 phenotype contributes to suppression of anti-tumor immunity and tumor growth. We have hypothesized that tumor growth can be effectively restricted by a special switch phenotype. The switch phenotype, in contrast to the M1 phenotype, should respond to anti-inflammatory pro-tumor cytokines by increasing production of pro-inflammatory anti-tumor cytokines. As a result, macrophages would be able to retain their anti-tumor features in the tumor area. We suggested this hypothesis after discovering human macrophages with characteristics of the switch phenotype. We showed in pilot experiments that production of pro-inflammatory cytokines by macrophages from patients with chronic obstructive pulmonary disease or bronchial asthma, in contrast to macrophages from healthy subjects, increased in response to anti-inflammatory stimuli. The phenotype we discovered was qualitatively different from the M1 and M2 phenotypes in the response to pro- or anti-inflammatory factors; therefore, we named this phenotype the M3 or the switch phenotype. Subsequently, a definition of the M3 phenotype was provided by Jackaman et al. defined as a phenotype with "incomplete polarization into an M1/M2-like phenotype". Despite different definitions of the phenotype, both Jackaman et al. and our group were referring to the same phenotype. The difference in these definitions was that we stressed the internal mechanism for emergence of such a phenotype (switching the signal from anti-inflammatory M2 factors to formation of a pro-inflammatory, M1 phenotype), whereas Jackaman et al. focused on external characteristics of the phenotype (the "M1/M2-like phenotype"). To use the M3 phenotype for restricting tumor growth, it was essential to learn the programming of this phenotype. Previously, we theoretically substantiated this possibility. Briefly, many reprogramming intracellular pathways branch to form an M1 or an M2 phenotype. For example, the TGF-β-dependent signaling can activate the M2 phenotype transcription factor, SMAD3, and the M1 phenotype transcription factors, NF-κB and p38. The JAK-dependent signaling can activate the M2 phenotype transcription factors, STAT3 and STAT6, and the M1 phenotype transcription factor, STAT1. In the tumor area, M1 macrophages activate the M2 phenotype pathway through anti-inflammatory cytokines, which results in activation of STAT3, STAT6, and SMAD3, increased production of anti-inflammatory cytokines, and reprogramming of the pro-tumor M2 phenotype. We suggested that inhibition of the M2 phenotype transcription factors, STAT3, STAT6, and/or SMAD3, could redirect the signal from anti-inflammatory, pro-tumor cytokines to activation of the M1 phenotype transcription factors. In this case, an M3 switch phenotype may form.

The aim of the study was to verify this hypothesis. Objectives of the study were to form an M3 switch phenotype *in vitro* and to evaluate the effect of M3 macrophages on growth of Ehrlich ascites carcinoma (EAC) *in vitro* and *in vivo*. We chose EAC as a tumor model because: 1) many abdominal tumors, such as pancreatic, ovarian, colorectal, and gastric malignancies, are associated with malignant ascites, and *2*) the murine EAC model is commonly used for evaluation of anti-tumor effects. After macrophage reprogramming, 25 000 EAC cells were added to activated M0 (Group 2), M1 (Group 4), $M3_{STAT3/6}$ (Group 6), $M3_{SMAD3}$ (Group 8), and $M3_{STAT3/6+SMAD3}$ (Group 10) macrophages at different ratios of macrophage number to EAC cell number, 5: 1, 10: 1, 20: 1, 40: 1, and 80: 1. An anti-tumor drug, cisplatin, was used at concentrations of 10, 20, and 40 μg/ml as a comparator. After co-culturing for 24 h, the content of cytokines in the medium was measured and the tumor cells were counted and their number was compared to that of tumor cells cultured without macrophages. Macrophages and tumor cells were separated after co-culturing according to the method described earlier. Briefly, macrophages, as distinct from tumor cells, became firmly fixed to the bottom of the wells. The plate was then vortexed, the content of the wells was pipetted, and all supernatant was collected. Cells of the supernatant were counted. These cells were represented mainly by tumor cells that did not adhere to the plastic. Cell culture experiments were performed in 5 replicates. A group of tumor cells cultured without macrophages was used as a control to tumor cells influenced by macrophages. The plate vortexing procedure left the number of dead cells almost unchanged; at the beginning of co-culturing and after 24 h of culturing followed by vortexing the plate, the number of dead cells did not exceed the range of 5–8% of total cell number.

Bottom Note: *This work is partly presented at* EuroSciCon Joint Event on Biotechnology, Stem Cell and Molecular Diagnostics April 16-17, 2018 Amsterdam, Netherlands.