The effects of blended medicinal mushroom preparations on survival and immunological and antimetastatic parameters in mouse CT26.WT colon cancer

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Introduction

• Colorectal carcinoma is one of the three most prevalent carcinomas in both males and females
• More common in highly developed industrialized countries
• Histopathologically most often adenocarcinomas
• Third most common cause of cancer-related deaths worldwide
• 40-50% die five years after diagnosis, despite advancements in treatment
• Hepatic metastases → 80% mortality
Introduction

• Local and systemic activation and regulation of immune system by malignant cells during carcinogenesis involves innate and acquired immune system

• Although malignant cells have antigenic properties their immunogenic effects are minor

• Tumor immune escape
  – MHC class I downregulation
  – Loss of co-stimulatory surface antigens
  – Decrease in apoptosis inducing death receptors (Fas/TRAIL receptor)
  – Loss of tumor infiltrating cytotoxic T cells by tumor induced apoptosis
  – Significantly disturbed cellular immune response
Introduction

- Macrophages are dynamic cells that modify their functional profiles according to various stimuli
- Terminology originated from differential macrophage (arginine) metabolism in mice with Th1 and Th2 profiles
- TAMs – similar profile as M2
- Th1 or Th2 induction promotes analogous M1 or M2 polarity
- Oversimplification

<table>
<thead>
<tr>
<th>Polarizing stimulus</th>
<th>M1: IFN-γ, LPS, IFN-γ+LPS</th>
<th>M2: IL-4, IL-13, Ic, IL-10, GC, GC+TGFβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenotype</td>
<td>Proinflammatory</td>
<td>Anti-inflammatory</td>
</tr>
<tr>
<td>In vitro morphology</td>
<td>Round/oval</td>
<td>Elongated, fibroblast-like</td>
</tr>
<tr>
<td>Products/Markers</td>
<td>TNFα, IL-1β, IL-6, IL-12, IL-23, CXCL10, pSTAT1, MMP9</td>
<td>IL-10, TGFβ, CCL17, CCL22, CD163, CD206, pSTAT3/6</td>
</tr>
<tr>
<td>Phagocytic activity</td>
<td>High</td>
<td>Low</td>
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<tr>
<td>Antigen presentation</td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Arginine metabolism</td>
<td>iNOS: Arginine -&gt; NO</td>
<td>Arg1: Arginine -&gt; Ornithine</td>
</tr>
<tr>
<td>Antibacterial capacity</td>
<td>High</td>
<td>Low</td>
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</tbody>
</table>
Introduction

• MMPs (matrix metalloproteinases)
• Roles in cancer progression
  1. Promotes tumor growth and survival
  2. Angiogenesis (VEGF, FGF, etc.)
  3. Invasion and EMT
  4. Inflammation and immune surveillance
Introduction

The 8 Hallmarks of Cancer

1. Sustaining Proliferative Signaling
2. Evading Growth Suppressors
3. Enabling Replicative Immortality
4. Resisting Cell Death
5. Inducing Angiogenesis
6. Activating Invasion and Metastasis
7. Reprogramming Energy Metabolism
8. Evading Immune Destruction
AIM of the study

- to examine the effects of two blended commercial medicinal mushroom extracts with or without standard chemotherapy used for treating colorectal cancer on various tumor parameters *in vivo*
Materials and Methods

• Balb/c mice, 2 to 3 months old, weighing 20-25 grams
• Standard conditions and diet
• Animal studies performed according to domestic and EU regulations
• **First set** of eight group of animals were inoculated with CT26.WT tumor cells, and after two weeks treated with 7 various combinations of medicinal mushroom preparations and 5-FU for another two weeks \(\rightarrow\) survival till day 55 after tumor inoculation observed
• **Second set** of eight groups of animals were inoculated with CT26.WT tumor cells, and after two weeks treated with 7 various combinations of medicinal mushroom preparations and 5-FU for another two weeks \(\rightarrow\) sacrificed and samples taken for analysis of various parameters
• \(n=10\) per group
Materials and Methods

• TESTED SAMPLES

→ Dr Myko San commercial blended mushroom products:
  
  **AGARIKON PLUS**; proprietary liquid extract blend of 10 mushroom species, including *Lentinus edodes*, *Ganoderma lucidum*, *Grifola frondosa* and *Agaricus brasiliensis* → dose 10 400 mg/kg (in saline), p.o. → AP

  **AGARIKON.1**; tablet form, 750 mg of polysaccharides per tablet

  *L. edodes*, *G. lucidum*, *A. brasiliensis*, *G. frondosa*, *Pleurotus ostreatus* → mortar and pestle → dose 1200 mg/kg (in saline), p.o. → AG

  and

  **FLUOROURACILUM (5-fluorouracil)**; Pliva, Zagreb, Croatia → dose 30 and 15 mg/kg, i.p. → 5-FU
Materials and Methods

• In both experiments:
• AGARIKON PLUS and/or AGARIKON.1 was administered **continuously** for two weeks in aforementioned dosages (260 mg/kg and 30 mg/kg respectively)
• 5-FLUOROURACIL was administered metronomically (**metronomic dosing**), the same way as in humans;
  1.-4. day (consecutively): 30 mg/kg
  6., 8., 10., 12. day: 15 mg/kg
• CONTROL given only saline p.o.
• Doses calculated by **interspecies allometric scaling**
Materials and Methods

• Treatment groups in both experiments as follows:

1. CONTROL
2. AGARIKON PLUS
3. AGARIKON PLUS + 5-FLUOROURACIL
4. AGARIKON.1
5. AGARIKON.1 + 5-FLUOROURACIL
6. AGARIKON PLUS + AGARIKON.1
7. AGARIKON PLUS + AGARIKON.1 + 5-FLUOROURACIL
8. 5-FLUOROURACIL
Materials and Methods

• CT26.WT (ATCC® CRL-2638™) – murine model of colorectal carcinoma, molecularly well characterized

• Undifferentiated Grade IV carcinoma – anaplastic (rare in humans)

• High tumorigenicity and tendency to metastasize, mainly in the lungs

• Cause high mortality inoculated to syngeneic Balb/c mice

• We inoculated 1x10^6 CT26.WT cells/mouse

• The treatment period was pre-fixed (two weeks after tumor inoculation), not modelled on changes in tumor volume! → simulation of advanced human colorectal carcinoma!!!
Materials and Methods

- First set of animals → survival
- Second set of animals sacrificed 14 days after tumor inoculation
- Spleens dissected and filtered through 40 µm nylon strainer, cell suspensions diluted 1:2 with DMEM and layered on Lymphoprep
- Macrophage supernatant obtained
  1. **NO concentration** established by Griess colorimetric assay kit (Promega, USA); optical density converted to µM of nitrites using standard curve for sodium nitrite
  2. **Arginase 1** activity assay; results calculated from the standard curve of urea and arginase activity and expressed as µM of urea
Materials and Methods

• Mouse serum obtained by centrifugation used for ELISA measurements of
  1. MMP-2
  2. MMP-9
  3. VEGF
  4. Th1, Th2, Th17 cytokine profiles
• Tumor tissue embedded in paraffin for establishing necrosis, number of blood vessels and number of mitoses
Materials and Methods

• Survival analysis
• Results were expressed by Kaplan-Meier plot
• Log rank test was made to compare the differences in survival time
• Survival analysis; 2 preventive groups
• Performed separately; LENTIFOM (LF) and AGARIKON.1 (AG) groups
• Pretreatment with LF and AG lasted for a week (same doses), and for another week after tumor inoculation
• Survival observed till day 45. after tumor inoculation
Results
Concentration of NO in supernatant of splenic macrophages

![Concentration of NO in supernatant of splenic macrophages](image-url)
Arginase activity
Th1/Th2/Th17 cytokine concentrations (pg/mL)
MMP-9 concentration (ng/mL)
MMP-2 concentration (ng/mL)
VEGF concentrations (pg/mL)
Tumor volumes (mm³)

Tumor volume (mm³)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1. measurement</th>
<th>2. measurement</th>
<th>3. measurement</th>
<th>4. measurement</th>
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<tbody>
<tr>
<td>CONTROL</td>
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<td>AP</td>
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<tr>
<td>AP + SFU</td>
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<td>AG</td>
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<td>AG + SFU</td>
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<td>SFU</td>
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</table>
Survival analysis: C vs AP

- $\chi^2 = 0.57$
- $p = 0.4509$
- Survival rate:
  - Control: 0/8
  - AP: 2/9
Survival analysis: C vs AP + 5-FU

- $\chi^2 = 8.92$
- $p = 0.0028$
Survival analysis: C vs AG

- $\chi^2 = 4.61$
- $p = 0.0318$
Survival analysis: C vs AG + 5-FU

- $\chi^2 = 7.56$
- $p = 0.0060$
Survival analysis: AP + AG

- $\chi^2 = 6.87$
- $p = 0.0088$
Survival analysis: AP + AG + 5-FU

- $\chi^2 = 11.71$
- $p = 0.0006$
• $\chi^2 = 7.35$
• $p = 0.0067$
Overall survival rates

1. CONTROL: 0/8 = 0%
2. AGARIKON PLUS: 2/9 = 22.2%
3. AGARIKON PLUS + 5-FLUOROURACIL: 7/8 = 87.5%
4. AGARIKON.1: 4/8 = 50%
5. AGARIKON.1 + 5-FLUOROURACIL: 3/9 = 33.3%
6. AGARIKON PLUS + AGARIKON.1: 5/9 = 55.5%
7. AGARIKON PLUS + AGARIKON.1 + 5-FLUOROURACIL: 5/10 = 50%
8. 5-FLUOROURACIL: 7/9 = 77.7%
Survival analysis: preventive groups

- In both preventive groups (n=10), ALL animals were still alive at day 45. post tumor implantation!!!
- According to literature no animals are alive after day 34 ± 6,2 days post CT26.WT tumor implantation
Conclusions

- Some polysaccharides are the best known and most potent mushroom-derived antitumor and immunomodulating substances.
- Polyphenolic compounds with antioxidant activity is also correlated with higher antitumor activity for some tumor cell lines.
- There is a known functional link between antioxidative effect and:
  - Increase in lifespan
  - Inhibition of ROS production necessary for tumor growth
  - Inhibition of VEGF
  - Activities of M1 and M2 macrophages (NO and arginase)
  - Macrophage polarization in favour of M1 tumoricidal efficacy
Conclusions

• Active compounds from medicinal mushrooms have significant antitumor effects in a dose dependent manner and can serve as biological drugs.

• That is especially relevant for patients with advanced metastatic and/or recurrent cancers, because in practice a lot of diagnoses are still made in late stages of a disease.

• Medicinal mushroom derivates are also helpful in patients with poorly differentiated and non differentiated tumors which are extremely life threatening and resistant to standard oncological therapies.
Conclusions

• This research has shown that even in advanced undifferentiated tumors, use of medicinal mushrooms can:
  1. Significantly increase overall survival rate
  2. Increase life span in percentages which are analogous to many human months
  3. Preventive groups have shown that the use of efficient doses of LENTIFOM and AGARIKON.1 can significantly prolong life span in the case of cancer occurrence
Thank you for your attention!

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