Immunological model

FD is a pro-inflammatory method which in many ways reverses immunossuppressive development of cancer disease by exposing tumour tissue to anoxia. There are at first presented some studies showing changes in tumour and cells which accompany hypoxia or anoxia and the immunological model itself follows.
The most important contribution of immunoediting hypothesis for FD is understanding cancer as a immunosuppressive insult spreading from primary tumour in time and space (61). Another interesting finding is that immunosupressive changes precedes nodal metastases (62) and that this immunosupressive preconditioning is orchestrated by the presence of tolerogenic DCs (63).

An inevitable result of these findings is the possibility that if there would be a proper pro-inflammatory insult which would change Th2 tumor protective polarization of immune system which is induced by tumor tissue in favour to Th1 polarization then immunosuppressive influence of the tumour would be diminished and this change would lead to the removal of the tumour. Thus cancer therapy can be viewed as a search for a proper pro-inflammatory insult.

The insult should contain all the known and unknown TAAs of primary tumour and its metastases, it should last for a sufficient time and have enough strength to induce long lasting specific antitumor immunity and at the same time its influence should be regulated to prevent SIR.

There should be defined quality and regulatory function of such a tumour specific pro-inflammatory insult.
The quality function of the tumor specific pro-inflammatory insult (TSPI) means quantity, immunogenity of TAAs, quality of TAAs presentation to effector cells and the fact that pro-inflammatory insults should contain as many known and unknown TAAs of primary tumour and all its metastases as possible. The quality function of TSPI is guaranteed by internal changes which occur inside anoxic tumour tissue in the case of FD.
The regulatory function of TSPI means the ability to secure the effect of the quality function of tumour specific pro-inflammatory insult in a range that guarantees a proper stimulation of the immune system and at the same time protects against SIR.
The regulatory function of TSPI is guaranteed by the presence of small areas among overlapping matraces sutures in FD. These tiny spaces and the pressure among them must be such that they enable the transfer of effector cells of the immune system and DAMPs inside and outside hypoxic department and at the same time the pressure must be high enough not to allow creation of free spaces among endothelial cells which would otherwise enable revascularisation. Too high a pressure among sutures causes release of hypoxic department and its disintegration and this way dilution of quality function of TSPI and improper and in most cases insufficient stimulation of the immune system. There have been not reported a single case of SIR in connection to FD.
The effect of both of these constituents – quality and regulatory function of TSPI is then modified by the conditions which regulate the immune system in general such as biobehavioral influences, status of metabolism etc.
As mentioned above the quality function of TSPI depends on the processes which take place inside hypoxic compartments.
Hypoxic department is presented by ligated tumor tissue in the case of FD. Tumor tissue contains two distinct cell types – transformed and non transformed. Transformed tumour cells undergo necrosis under hypoxic conditions and non transformed stromal cells undergo apoptosis.
It has been shown that 10-15% of genes change their expression 15 minutes after tissue resection and that this share rises to 20% after another 15 minutes in healthy and malignant

colon tissue. Investigation of protein profiles found that the intensity of overall 30% of protein peaks changed significantly during 30 minutes and most changes happened during the first 15 minutes after resection. The study included results from samples taken from the central area of the tumour only because a variability of protein profiles of 40% among samples taken from periphery to central area was found. Gene expression changes effect all functional gene groups including genes connected with hypoxia such as HIF-1alfa, cytoskeletal genes and genes which seems to have antiapoptic function such as CEA (64). Different susceptibility to hypoxia can be explained by the fact that there is a gradient of hypoxia present in tumour tissue under normal circumstances which lowers O2 partial pressure as low as to 5-10 mmHg in some areas of the tumour (65).

The presence of hypoxic areas within the tumour has a profound impact on health and tumour cell physiology. Hypoxia induced genomic changes can be detected at O2 partial pressure of less than 1 mmHg. It has been found a 3.4 fold times higher mutation rate in mouse tumour cells line carrying chromosomal based lambda phage shuttle vector cultured in hypoxia of 1 mmHg after 4 hours to cells cultured in normoxia. Hypoxia represents a strong selective pressure which selects neoplastic cells with apoptic insensitivity and increased angiogenic potential which contributes to tumour malignancy (66). This finding has been supported by Park's study which investigated changes in the behaviour of tumour cells under hypoxic conditions. Hypoxia in tumour leads to a decrease in cell adhesion by activating proteolytic system of MMPs. This process is believed to free tumour cells from cross linked I type collagen network of extracellular matrix. This activation is accompanied by an increased production of MMP-13 and its enhancers such as cytokines S100A4, IL-1, VEGF, PDGF-BB as well as a decrease in TGF- beta. Another change found is an increase in angiogenic factors: ANG, VEGF, IL-1, IL-3, GRO-alfa, PDGF-BB with an even further increase in PDGF-BB, GM-CSF with prolonged hypoxia. An increase in potent chemoattractants G-CSF and GM-CSF for macrophages and neutrophils is thought to serve for the recruitment of these cells and their secretion contributes to further destruction of ECM. Moreover, some types of tumour cells are capable of secreting further chemokines as stroma derived factor-1 and CXCL-16. All these changes explain increased invasiveness and a metastatic potential for tumour cells under hypoxia conditions (67).

An interesting study about the survival of tumour cell lines under anoxi was done. Examination of the survival of 22 cancer cell lines of various origin cultured under anoxia showed 80% to 100% survival in 11 cell lines after 4 days, 2 after 3 days, 3 after 2 days, 6 after 1 day (68).

A change in expression of TAPP has been investigated in glioma cells. The findings were an increased expression of 9 glioma TAPP and a decrease in 2 out of 25 investigated TAPP in glioma cells cultured in hypoxia of 1% in comparison to glioma cells cultured in normoxia (69).

Hypoxia influences also non tumorous cells. DCs maturating under hypoxic conditions of 1% O2 partial pressure compared to Dcs maturing under normoxia 20% O2 partial pressure up-regulate genes encoding various members of pattern recognition receptors, scavenger receptors CD36 and APOB48R, adhesion/homing receptors, Ig-like immunoregulatory receptor family, Ig-Fc receptors. All these changes indicate higher inflammatory functions of H-mDCs, their increased capacity to migrate to secondary lymphoid organs, higher ability of activating adaptive and innate immune response in different ways. Hypoxia also influences interaction of DCs and other effector cells of immune system including regulation of myeloid cell functions. In addition H-mDCs seems to contribute to atherosclerosis (70).

Stimulation of mouse CD4+ T cells under hypoxic conditions of partial O2 pressure of 1% results in an increased production of CD4+ T cell cytokines especially IFN- gamma in comparison to activation under normoxia (71).

Low tissue oxygenation drives the ability of some cells to react to further hypoxia. It can be illustrated with the case of tumour associated macrophages and monocytes. Human

monocytes are not capable of inducing expression of HIF-1 alfa or HIF-2 alfa under hypoxia contrary to human monocyte-derived macrophages. TAM which are present within the tumour show a high expression of HIF-1alfa and HIF-2 alfa. hMDM which were previously exposed to chronic hypoxia induce higher levels of HIF-1 alfa and HIF-1 alfa than those exposed to normoxia or acute hypoxia.

Changes in metabolism in hypoxia must be considered as well. Krebs cycle as a source for ATP is knocked out and replaced by glycolysis. Glycolysis leads to accumulation of lactate inside cells which causes cellular acidosis. There is an increased production of ROS and peroxidation of lipids which lead to membrane damage under hypoxia. Necrosis and increase in tissue acidosis are factors which recruit more leukocytes (65).

It must be taken in consideration when evaluating the below mentioned processes in connection to the cellular death that despite the fact that tumour is an immunosuppressive agent there are dormant pro-inflammatory components inside it which can be potentially activated at least for some time if immunossupressive influence diminishes.

This can be demonstrated in the case of TAM which are in most studies supposed to be indicator of poor prognosis contributing to tumor progression but there are pro-inflammatory macrophages, macrophages with some signs of both pro-inflammatory and immunosupressive activation and macrophages presenting immunosupressive signs within the tumour. Their amount differs according to the type of the tumour, stage of disease, localisation within the tumour and signals received from microenviroment (65,72).

Three ways of cell death are distinguished: acute apoptosis, late apoptosis and necrosis (73). Apoptotic cells according to hidden-self model do not cause inflammation since their intracellular immunostimulatory molecules are coated by an impermeable membrane and these vesicles are cleared by phagocytosis (74). If apoptotic vesicles are not cleared fast enough the membrane becomes permeable and becomes immunostimulatory. This stage of cell death is called late apoptosis. Necrotic cells are immunostimulatory since they do not cover their intracellular content by a membrane after their death.

Early apoptotic cells are not passive entities but they actively attract phagocytes by release of chemotatic factors such as LPC, EMAP II and fractalkine/CXC3CL1 which are supposed to recruit macrophages and monocytes. Migration of neutrophils is inhibited by a release of lactoferrin. Phagocytosis is ensured by changes in apoptic cells which include the presence of ACAMPs, lost of don't-eat me signals as CD47, SIRP-alfa, CD31, loss of phospholipid asymetry of cellular membrane associated with exposure of anionic phospholipids.

Late apoptotic cells lose membrane integrity and so expose intracellular molecules. Necrotic cells do expose intracellular molecules per primam. (73). Among these intracellular molecules associated with death cell are HSPs, uric acid, HMGB1, genomic dsDNA which are ranked among DAMPs (74). All these changes lead to a complex activation of specific and non specific immunity response due to activation of classical complement pathway, manose binding lectin, ficolin-2 and ficolin-3, properdin, pentraxin family, histine rich glycoprotein, trombospondin-1, heparan sulphate proteoglycans, antibody (73).

It has been shown that TLRs a key component of innate immunity can be stimulated not only by microbial antigent but also by phagosomes of tumour cells. This activation leads to expression of tumour antigen on the MHC class II on APCs and can later activate antigen specific T lymphocytes. Stimulation of TLR8 by poly C3 and ssRNA compounds markedly attenuates regulatory Foxp3+ T cells activity. (75)

HSPs are proteins with intracellular chaperone activity. They represent 1-2% of total protein content in non stressed cells which increases to 4-6% under stress conditions (76). They are products of different gene families. The Hsp70 families produce major stress inducible Hsp70s proteins that are together often referred to as Hsp72, Hsc70, Grp78, Hsp110. The HSP90 family produces Hsp90a and Hsp90b and Grp94. And there has been also mitochondrial Hsp60 protein (77) Hsp27 family is represented by HSP27 ATP dependent protein which is located in cytosol. It is necessary to mention the gp96 protein sited in endoplasmatc reticulum.

HSP interacts with many proteins for example HSP90 has more than 200 clients proteins such as HIF-1 alfa, VEGFR, HER2, BCL-ABL, SRC, androgen, estrogen receptors and others (76). HSPs are found in tumour in exosomes in a free form or associated with surfaces of tumour cells. They maintain immunossuppressive as well as immunostimulatory functions. The way they act depends on the microenviroment they are in - immunosuppressive or immunostimulatory and their form as being coated they perform immunosuppresive functions. (77) HSPs differ in their immunogenity. It has been presented that in sarcoma model immunogenity of hsp90 was approximately 10% of that of gp 96 or hsp70. There are two ways the HSPs induce antitumor immunity. HSPs can induce presentation of antigen peptide on MHC I class molecules of APC and this way activate CD8+ T cell response if being a part of HSP-chaperoned antigenic peptide complex or HSP in the absence of antigenic, tumour derived peptides they can activate NF-kB pathway by interaction with signalling receptors e.g. TLR2, TLR4, CD14 thus inducing non specific stimulation of the innate immune system by production of pro-inflammatory cytokines e.g. IL-1 beta, IL-6, TNF-α. (76) HSPs are also capable of inducing maturation of DCs after overreaching critical concentration. It has been proposed that this concentration can be reached by inducing necrosis of tumour cells in vivo. It has been estimated that necrosis of 1 mg of tumour tissue (10 up 5 to 10 up 6 cells) will release about 2 ug of total hsps in a volume of 1-2 ul or 1-2 mg/ml which really provides sufficient concentration of HSPs to overreach this threshold. (77) The moreover there is indication that HSPs are able to directly present antigent through the MHC II class molecules and that they may stimulate T helper cells. (77) Autologous melanoma derived heat shock protein gp96-peptides complexes induced clinical and tumour specific T-cell response in a significant minority of patients during clinical trials performed by Belli without significant toxicity. (79). It has been demonstrated that induced necrosis of normal melanocytes in the presence of elevated levels of Hsp70 induces CD8(+) T-cell-dependent, antigen-specific response in mice that eradicated systemically established B16 tumours (80).

Inflammation is closely related to fibrosis. Neutrophils at the site of inflammation undergo activation with respiratory burst that produces reactive oxygen species, release serine proteases as elastases and cathepsin G. (81). Reactive oxygen species, signs of stress, dying cells, the presence of microbes, paracrine cues from neighbouring cell types stimulates fibroblasts. Macrophages also stimulate fibroblasts by production of TGF-beta, PDGF and induces production of TIMPs which block ECM degradation. On the other hand, activated fibroblasts stimulate macrophages by production of MCP-1, M-CSF and other chemokines. The final amount of fibrotic tissue depends on the way macrophages are activated. If macrophages are classically activated by Th1 cells producing IFNγ then fibrosis is impaired if macrophages are activated by Th2 cells, then alternatively activated macrophages support fibrosis production (81). The small amount of fibrotic tissue in devascularised compartment in FD treated tumours indicate that macrophages are classically activated by Th1 cells.

There is of note that each tumour represents a unique accumulation of mutant sequences with novel antigen epitopes which creates specific 'antigenic fingerprint' of each individual tumour (80). The „antigenic fingerprint" and gene expression does not change itself only among individual tumours but also among primary tumour and its metastasis and that metastatic cancer shows different immunohistochemical phenotype according to metastatic site (83; 84).

Immunogenity of tumour lysate cultured in hypoxia of 5% partial O2 pressure and normoxia of 20% partial O2 pressure has been compared in glioma cells. Higher immunogenity of hypoxic lysate with gene expression more similar to tumours in situ and higher expression of known immunogenic antigens have been established. An intrinsic adjuvant activity in hypoxic TL independent to TAA expression has been shown (87).

The comparison of immunogenity among primary tumour and between metastases has not been investigated to author's knowledge. It could be reasonably expected that primary tumour immunogenity is higher than that of it metastases or metastases originated from metastases due to the selective pressure made by the immune system during the process of

founding metastases.
In evaluating the effectiveness of FD it must be taken into account that histologically the same tumour can substantially differ in immunogenity and the ability to induce Th1 or Th2 type immunity response as being demonstrated in LR and LS lymphoma inoculated mice (86).
Curability of FD may also be influenced by factors which influence responsiveness of the immune system in general such as biobehavioral influences, operation management (87), stage of the disease and status of metabolism or status of hemopoiesis.
Biobehavioral influences and their impacts on the immune system are discussed elsewhere (87).
Involment of hemopoiesis is supported by a finding of the increase in production of G-CSF and GM-CSF during hypoxia (67). It can be reasonably supposed that newly recruited macrophages and neutrophils will support pro-inflammatory response in an environment containing leaking debris from ligated tumor tissue.
All above mentioned changes in connection to exposure of tumor tissue to anoxia conditions indicates increase in quantity of TSPI by incresed expresion of TAA, activation of HSP pathway and activation of cellular immunity.

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