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Autologous tumor immunizing devascularization in cancer therapy

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ABSTRACT

Tumor vaccination depending on specific antigens, autologous tumor vaccination involving wide range of antigens, immunomodulating cytokines and bacterial agents have been studied extensively with the purpose of stimulating the antitumor immune response. Unfortunately these therapies showed disappointing results mainly due to undesirable mechanisms tending to dampen the antitumor immune response. We will discuss a novel approach of autologous tumor immunization using a surgical technique: autologous tumor immunizing devascularization (ATID). This approach involves complete surgical devascularization of a tumor which is then left isolated *in situ* in the body. The stressing pathophysiological condition of the completely isolated tumor provokes a generalized immune response which, as shown from clinical cases, leads to the elimination of the devascularized tumor and distant metastases without causing sepsis. Until now no clinical study was properly executed. The possible significance of this method which resides in its curative potential has thus escaped attention in the field of cancer therapy. This article will hypothesize optimal physiological criteria and necessary clinical conditions for ATID to be performed effectively. The main criteria are (1) complete isolation of the tumor from the vascular system, (2) sufficient devascularized tumor load to trigger a sustained generalized immune response to cancer antigens until elimination of all cancer loci, (3) tumor cell killing rate corresponding to the elicited immune response is higher than the tumor cell growth rate, and (4) patients with an uncompromised immune system. Future studies have to be performed under the indicated conditions in order to confirm the efficacy and safety of ATID as a novel approach in the treatment of cancer.

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Background

In tumor immunotherapy many techniques have been developed to stimulate the immune system against tumor specific antigens in order to reduce or eliminate the tumor. The effect of tumor vaccination has been disappointing, most probably due to the difficulty of identifying and appropriately implementing tumor-specific antigens in order to produce a sustained immune response against tumor cells [1]. Also whole cell tumor antigen vaccines, with many potential tumor antigens, did not have the desired clinical effect, probably due to the immune system's inherent tolerance to several antigens expressed in the whole tumor cell preparation, as they may be expressed by normal tissues or presented to T cells in a non-stimulatory context [1]. Also tumor microenvironment is replete with mechanisms that dampen antitumor immune responses locally [2]. Even combined with immune stimulatory molecules whole tumor cell vaccines might have negative impact on immunomodulation and outcomes [3]. A new surgical technique might overcome the problems of immune tolerance and elicit a non-toxic generalized immune response to tumor antigens. This technique is based on complete surgical devascularization of a tumor which is then left isolated *in situ* in the body. The stressing pathophysiological condition of the completely isolated tumor provokes a generalized immune response which leads to the elimination of the devascularized tumor and distant metastases without causing sepsis [4–14].

This new surgical technique was found out by coincidence. In 1957, the Czech surgeon Karel Fortýn encountered, during an emergency operation of a middle-aged man wounded by gunfire, a metastasized inoperable stomach tumor. To prolong the life of his patient he arrested all hemorrhages, also closing ruptured arteries and veins from the stomach tumor and performed a jejunal stomach bypass. Due to the extent of the tumor, only a palliative procedure was possible, in which a large part of the totally devascularized stomach remained *in situ*. This procedure at that time had no known clinical precedent. Unexpectedly, the patient recovered without complications and surprisingly the metastasizing tumor regressed completely with no relapse after years of follow-up. After this event, Dr. Fortýn performed the same technique, he called tumor devitalization, in patients mainly with metastasized

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carcinomas of the colon, stomach and kidney with the same results [4–6]. Later animal studies indicated the working principle of tumor devitalization, resulting in the activation of the cellular immune system against tumor antigens [7–14]. To point out the immunological mechanism of the procedure we renamed the technique: autologous tumor immunizing devascularization (ATID). The conducted studies on ATID contradict an important long standing surgical dogma and provide manifest evidence of the existence of two fundamental immunological principles.

The contradicted dogma is that an important volume of devascularized tissue completely isolated from all sources of oxygen and nutrients can be left *in situ* in the body without any serious consequences. It is known that partial arterial devascularization by means of embolization can create a post-embolization syndrome resulting in fever, nausea/vomiting, pain and sometimes sepsis, due to the release of chemical mediators systemically [15–17]. This does not occur following complete tumor devascularization when besides arterial flow also venous reflux and lymph flow are arrested [7].

The first immunological principle involved in ATID is the promotion of apoptotic mechanisms and the safe progressive elimination of devascularized either healthy tissue or tumor mass which is replaced in a varying extent by a residue of fibrous tissue [4]. This process is in contrast to necrosis in which cellular mass is destroyed indiscriminately by liberated intracellular lytic enzymes. The second immunological effect of ATID following the devascularization of a tumor is triggering of a generalized systemic immune response to tumor specific antigens so that vicinal as well as distant metastases are also eliminated. This contrasts with tumor embolization after which no reduction of distant metastases occurs [18]. This process evoked with ATID we call immunolysis: the genetically controlled biomolecular degradation by the immune system of the devascularized healthy tissue and/or tumor mass, including possible existing metastases.

Safety and elimination of both primary devascularized tumor and distant metastases by ATID have been demonstrated in animal studies [7–14], as well as human case series [4–6,19]. Safety of ATID was also verified in an officially authorized multicentre clinical trial [20]. However, the therapeutic efficacy was not confirmed, because fundamental principles and clinical indications were not respected: uncompromised immune system and a sufficient volume of devascularized tumor to evoke a generalized immune response against tumor specific antigens (TSA) and tumor associated antigens (TAA).

Hypotheses

To perform curative surgery with the ATID technique, we hypothesize that several conditions should be met:

(1) Not only arteries but also veins should be interrupted to evoke a generalized immune response against TSA and TAA.
(2) Minimum volume of devascularized tumor is required in relation to the remaining volume of non-devascularized tumor tissue to obtain a sustained activation of the immune system until elimination of all cancer loci.
(3) The tumor cell killing rate corresponding with the elicited immune response must be higher than the unhindered tumor cell growth rate.
(4) Uncompromised immune system.

The four hypotheses will be hereunder elucidated.

Complete isolation from the vascular system

Tumor embolization is the interruption of the arterial tumor blood supply leading to tumor ischemia, with the intention to reduce the tumor size. This technique is mainly employed in the liver to reduce liver metastases, though no reduction of distant metastases have been observed. The fundamental difference between tumor ischemia due to embolization and ATID is that in the latter case also the venous reflux and lymph flow are interrupted. The main differences between ischemia and ATID in physiological and biomolecular effects are the following:

(1) After embolization, prolonged anaerobic glycolysis leads to massive necrosis because apoptotic signaling mechanisms are progressively disabled by the lowering of extracellular and intracellular pH and by the cellular influx of Ca^{2+}, while
(2) in ATID, the acute cut-off of all nutrients promotes apoptosis following the rapid depletion of ATP energy reserves due to inhibition of prolonged anaerobiosis.
(3) After embolization necrosis leads to indiscriminate proteolysis of potential antigens, while
(4) in ATID phagocytosis of apoptotic cellular debris allows normal processing of immunogenic amino acid sequences by antigen presenting cells (APC) for presentation to T-lymphocytes and thus enables the triggering of a systemic immune response to TSA and TAA. This does not occur after destructive necrosis.

These differences are explained by the sequence of events as they occur in (a) embolization and (b) ATID respectively:

(a) In embolization, when only arterial blood flow is interrupted the critical limiting factor is the supply of oxygen while other blood components are still available through venous reflux and continuing lymph flow. When oxygen availability is limited, while the supply of glucose and other nutrients is still adequate, the cellular reaction is the onset of anaerobic glycolysis to support critical energy requirements of cellular metabolism [21]. This normally occurs in emergency situations, for instance, the arrest of hemorrhage by arterial ligature. Ischemia under such conditions can only be maintained for a very limited time.

The critical pathophysiological state is caused by the decreasing pH of interstitial fluid, due to the increasing concentration of CO_2 from residual respiration and lactate produced during anaerobic glycolysis.

Firstly, the decreasing extracellular pH tends to increase the concentration of Ca^{2+} normally bound to plasma proteins [22].

Secondly, as cellular ATP energy reserves are progressively depleted, cell membrane potentials controlling critical transport functions are negatively affected by the increasing acidification so that ATP dependent Ca^{2+}ATPase and other calcium transport functions cease to operate adequately [23]. Intracellular Ca^{2+} concentration thus tends to increase rapidly to prohibitive levels due to disabled active transport function of Ca^{2+}ATPase, normally operating against an enormous concentration gradient of 1:10 000. The result is that proper intracellular signaling is inhibited since the critical functions are influenced by only very small differences in Ca^{2+} concentrations [24]. Apoptotic mechanisms are dependent on these signaling functions and normally assure the controlled and safe elimination of endangered cells through apoptotic granulation. In case of ischemia and prolonged anaerobiosis these apoptotic mechanisms are abruptly disabled and cannot prevent the onset of necrotic lysis. Furthermore, phospholipases tend to be excessively activated resulting in the disruption of intracellular vesicular membrane integrity with liberation of lytic components which contribute to final necrotic degradation of the cellular mass followed by the release of dangerous mediators in the blood stream [25]. In the ensuing catabolic process, indiscriminate proteolysis destroys immunogenic amino acid sequences of TSA and TAA. The possibility of triggering a generalized immune response is thus compromised because potential tumor antigens must not

be denatured for immunogenic presentation by APC and activation of cytotoxic T-lymphocytes (CTL) to become effector CTL (ECTL) [26].

(b) In ATID, the pathophysiological condition of total starvation is produced when the tumor is completely devascularized and besides arterial interruption also venous reflux and lymph flow are arrested. Thus, with the onset of ischemia and interruption of the supply of glucose and of all vital growth factors, normal functional metabolic activity comes to a halt as cellular ATP reserves are progressively depleted. This pathophysiological condition promotes apoptotic granulation since prolonged detrimental anaerobic glycolysis is inhibited. In this phase, in order to prevent damaging catabolic degradation, the defense reaction of the organism is to preserve cellular integrity by avoiding the disruption of cellular membrane structures and thus dangerous necrosis which can lead to anaphylaxis through massive leakage of inflammatory mediators into systemic circulation [27]. The reaction may be seen as a natural manifestation of the biological principle of survival in which a controlled limited local sacrifice protects the entire organism [28,29]. Apoptotic cellular fragmentation allows progressive and controlled resorption of the endangered cellular mass by phagocytosis of organic debris as in the normal elimination of dying cells in the life span of the organism. From the beginning, when ATP reserves are not yet completely depleted, the overall situation is monitored by signaling molecules involving the whole family of stress signaling factors (SSF), especially, heat shock proteins (HSP), particularly HSP90 and HSP70. Their concentrations are increased following ATID and during the apoptotic process they slowly diffuse into the systemic circulation [13,30]. This is the signaling phase in the mobilization of the immune system attracting leucocytes to the site of intervention. There, phagocytizing APC slowly resorb apoptotic cellular debris. Immunogenic non-self amino acid sequences which are preserved in the apoptotic debris can thus be regularly processed for antigen presentation to CTL [26].

Thus, in the process of immunolysis, the biochemical identity of non-self amino acid sequences tends to be preserved mainly by the granulation of the cellular mass and with lymph and venous drain arrested no pro-inflammatory mediators are massively released systemically [7]. This effect in ATID is in contrast with embolization leading to post-embolization syndrome. Also tumor radiotherapy with rapid necrotic decay of tumor cells can lead to the typical "lysis syndrome" [31].

When large volumes of cellular tumor debris following ATID are phagocytized and the signaling and specific activation is sufficiently strong, the probability of APC presenting potentially immunogenic TSA and TAA is greatly enhanced. The massive generation of promoting mediators then produces a reaction cascade which finally triggers a generalized immune response to TSA and TAA. This mechanism is a *multimodal activation* and its specificity resides in the multiplicity of immunogenic factors and potentiating mediators all interacting conjointly *in vivo* as they are generated systemically [2,32].

In ATID therefore, as shown schematically in Fig. 1, we have to consider the following critical steps: (1) pathophysiological stress in a sufficiently large volume of malignant tissue, (2) signaling and immune system mobilization phase with the generation and release of HSP and other SSF components (3) ATID induced apoptosis in cells undergoing pathophysiological stress, (4) phagocytosis by APC of apoptotic debris, (5) interaction of T-lymphocytes with HSP/TSA and HSP/TAA complexes, (6) generation and activation of precursor CTL and (7) finally the activation of ECTL through CD28 and CD80 co-stimulation by APC presenting the totality of TSA and TAA.

The above sequence of events is necessary to obtain the massive activation of effector cytotoxic T-lymphocytes and other

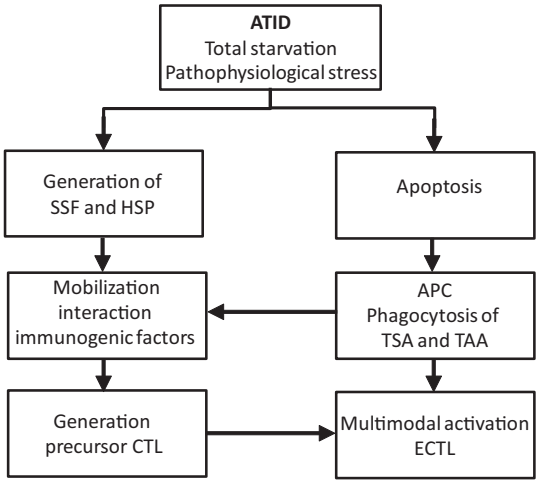

Fig. 1. Critical multimodal activation steps in ATID, resulting in tumor immunolysis. ATID: autologous tumor immunizing devascularization, SSF: stress signaling factors, HSP: heat shock proteins, APC: antigen presenting cells, TSA: tumor specific antigens, TSA: tumor associated antigens, CTL: cytotoxic T-lymphocytes, ECTL: effector cytotoxic T-lymphocytes.

cooperating immune system components in an immune response cascade effective against non-devascularized metastases. These physiological conditions are not obtained in the case of simple embolization.

Optimum volume of the devascularized tumor

Following ATID the systemic specific cytotoxic activity against tumor cells is maintained until complete resorption of the devascularized tumor mass. The level of immunolytic activity against tumor cells is therefore dependent on the quantity of the devascularized mass and will tend to diminish as the residual devascularized malignancy is progressively immunolysed [33]. If the initial volume of devascularized tumor is sufficiently large the systemic specific cytotoxic activity first attains a maximum natural value which will begin to decline once the natural decay rate of ECTL and cooperating components is greater than their rate of generation and activation [33]. All these reactions proceed under immunologically controlled conditions to avoid damage to healthy tissues if the targeting of tumor cells by the immune system were not to be appropriately specific [34].

The prevailing cytotoxic activity, named as effective killing rate (K_{ef}) is a variable value depending on numerous immune activation functions which determine the systemic concentration of CTL (L). K_{ef} is either equal or inferior to the corresponding maximum killing rate (K_{max}) for either tumor or healthy cells.

Both values can be expressed as the number of cells (N) killed per cell per hour [h^{-1}] and are defined by the differential rate formula $dN/dt = K_{max}N$. Its integration for the time interval $\Delta t = t_2 - t_1$ leads to the logarithmic relationships:

$$K_{max} = -\ln(Nt_2/Nt_1)/(t_2 - t_1)[h^{-1}] \quad (1)$$

$$Nt_2 = Nt_1 e^{-K\max(t2-t1)} \quad (2)$$

K_{max} applies equally for every separate tumor locus in the body. On the other hand, the effective growth rates of tumor cells (G_{ef}) defined in the same way as K_{max} by $dN/dt = G_{ef}N$ are different in all non-devascularized tumor loci remaining in the body [35].

Therefore, if ATID is to be curatively effective, the critical condition is to assure $K_{ef} > G_{ef}$ at every tumor locus for a sufficiently long period allowing all tumor loci to be eliminated before the systemic cytotoxic activity falls to inadequate levels when $K_{ef} < G_{ef}$. This

condition can only be met if the devascularized mass of tumor cells with zero growth is greater than the residual mass of malignant cells which continues to grow unhindered by any physiological limitation. The necessary condition for the elimination of metastases is therefore expressed as the critical ratio of devascularization $Rd = D/T \gg 1$ where D is the mass of devascularized tumor and T is the residual mass of any freely growing primary tumor and metastases.

The critical Rd is not a constant and can only be estimated by model computation and simulation of K_{ef} on the basis of K_{max}, the systemic generation rate of ECTL (Lg), the decay rate of ECTL (Ld) and the observed tumor growth rates (G_{ef}). G_{ef} can be estimated by PET-scanning and varies from case to case depending on the type of cancer and the various stages of development of individual metastases [36].

When the model simulation of the immunolytic process is effected for given values D and T, G_{ef}, K_{max}, Lg_{max}, and Ld [33] we find that higher values of Rd may be necessary to eliminate completely larger individual metastases. However, lower values of Rd may be adequate in the case of even a large number yet smaller tumor loci, because these are eliminated in less time than a single large metastasis with an equal total mass. Thus, when metastases are larger, it is necessary to devascularize a larger tumor mass to prolong the activation phase of the immune system in order to maintain the systemic concentration of ECTL at the maximum possible level until elimination of all tumor loci.

The main limiting factor in the immunolytic process is K_{max} which determines the critical value Rd. We have estimated K_{max} using the results of the minipig animal model. By studying the process of immunolysis after devascularization [4,5], it was possible to estimate the value of K_{max} on the basis of the time required to obtain a given volume reduction of a devascularized mass of healthy tissue (colon segment, entire kidney, spleen) according to the relationship $K_{max} = -\ln (Nt_2/Nt_1)/(t_2 - t_1)$ (Eq. (1)) where N is the number of cells in the devascularized mass of tumor or healthy tissue undergoing immunolysis. The estimations indicate a mean empirical value of $K_{max} \sim 0.005–0.007\ h^{-1}$. On purely theoretical grounds, K_{max} was also estimated by computation using established physiological parameters presented in Table 1. The calculation is made on the basis of the simplifying condition taking tumor volume at the limit when the tumor surface for the infiltration of ECTL is not dependent on the degree of vascularization. By analogy, the limit tumor volume may be compared with the size of the embryo in early embryonic development when vital nutrient supply proceeds entirely by diffusion in the absence of vascularization.

V_0 is the limit volume of tumor when tumor radius $R = \sim 1.5$ mm, S is the effective tumor surface independent of vascularization and $N = V_o 10^6$ is the corresponding number of tumor cells.

Surprisingly, according to the relationship:

$$K_{max} = L_s S L_m K/N \quad [h^{-1}] \tag{3}$$

the calculated value $K_{max} \sim 0.005\ h^{-1}$ coincides with the estimated empirical value observed in animal studies in the immunolysis of healthy tissue. We can therefore assume that similar mechanisms are involved in the immunolysis of both healthy tissue and tumor mass following ATID.

Under clinical conditions, the precise estimation of tumor growth rate G_{ef} using the relationships Eqs. (4) and (5) may be difficult, because the small differences $\Delta N = Nt_2 - Nt_1$ in tumor mass in any reasonable time interval $\Delta t = t_2 - t_1$ are not adequately revealed when sonography, CT, or NMR are used as diagnostic imaging techniques.

$$Nt_2 = Nt_1 e^{Gef(t2-t1)} \tag{4}$$

$$G_{ef} = ln(Nt_2/Nt_1)/(t_2 - t_1)[h^{-1}] \tag{5}$$

However, better possibilities are offered by PET where precise differences in metabolic rates in given time intervals can be directly correlated with the growth rate, for instance, following the rate of assimilation of fluorine-18 labelled fluorodeoxyglucose [37].

The reduction of tumor volume and its graphical expression in individual plots of tumor mass over time for every diagnosed tumor locus can be calculated by using the parameters in Table 2 in a set of differential equations (Eqs. (6)–(9)) and activation functions (Eqs. (10)–(13)) following standard numerical computation methods [38]. By carrying out the computation for given values of D_0, T_0, M_0, Ld and Lg_{max} it is possible to determine the required value Rd for individual clinical cases. Actual computations show that in most situations Rd values of 5–10 are adequate.

In the model, the numerical computation of the physiological parameters D_t, T_t, M_t, L_t and K_{ef}, is expressed as a plot of the individual values in function of time.

The required or optimum value Rd is obtained by a series of computations on the basis of the initial values D_0, T_0 and M_0 that

Table 1
Physiological parameters in the calculation of K_{max} (Eq. (3)).

Symbol	Physiological parameter	Dimension
L_s	ETCL concentration at tumor surface	count per mm^3
L_m	ETCL migration rate in affected tissue	mm/h
V_o	Volume of tumor	mm^3
R	Mean radius of tumor	mm
S	Effective tumor surface	mm^2
K	Killing capacity: tumor cells killed per ECTL	
N	Number of tumor cells in volume of tumor V_o	

Table 2
Physiological parameters in model simulation of immunolytic process.

Symbol	Physiological parameter	Dimension
D_0	Devascularized mass of tumor	g
D_t	Residual mass of devascularized tumor after time t	g
T_0	Initial mass of non-devascularized primary tumor	g
T_t	Residual mass of non-devascularized primary tumor after time t	g
M_0	Initial mass of metastases	g
M_t	Residual mass of metastatic tissue after time t	g
L_i	Initial concentration in arbitrary units (u) of precursor CTL in devascularized mass of tumor[1]	u/g
L_0	Initial systemic concentration of precursor lymphocytes: L_i. D_0	u
Lg_{max}	Maximum generation rate of specific immunolytic ECTL	h^{-1}
L_{max}	Maximum concentration of specific immunolytic ECTL	u
L_t	Concentration of immunolytic ECTL after time t	u
L_{min}	Limit minimum concentration of immunolytic ECTL	u
Ld	Decay rate of specific immunolytic ECTL	h^{-1}
K_{max}	Maximum systemic immunolytic killing rate	h^{-1}
K_{ef}	Effective immunolytic killing rate in separate tumor loci	h^{-1}
G_{ef}	Effective growth rate of tumor cells in separate loci	h^{-1}
V_o	Effective limit volume of tumor in absence of vascularization	mm^3
C_m	Mean mass of tumor cell	g
m_0	Limit mass of tumor in absence of vascularisation: $V_o C_m 10^6$	g
NM_t	Number of cells in metastasis after time t: M_t/C_m	
NT_t	Number of cells in primary tumor after time t: T_t/C_m	
ND_t	Number of cells in devascularized tumor mass after time t: D_t/C_m	
t	Time	h

[1] The arbitrary unit u is taken equivalent to the maximum number of ECTL in 1 mm^3 of peripheral blood at the interface between tumor and vascular system. Ratio of devascularization $Rd = D_0/(T_0 + M_0)$.

allow complete elimination of tumor growth when the values NT_t and NM_t in certain time t are inferior to zero.

The numerical computation is effected using the following set of differential equations and activation functions:

Immunolytic killing rate equations

$$dD/dt = D_t(G_{ef}(D) - K_{ef}(D_t)) \quad (6)$$

$$dT/dt = T_t(G_{ef}(T) - K_{ef}(T_t)) \quad (7)$$

$$dM/dt = M_t(G_{ef}(M) - K_{ef}(M_t)) \quad (8)$$

$$dL/dt = L_t(Lg_t - Ld) \quad (9)$$

Activation functions

$$Lg_t = Lg_{max}(D_t/D_0)(1 - L_t/L_{max}) \quad (10)$$

The CTL generation rate is dependent on the residual quantity of devascularized tumor D_t and is controlled by feedback regulation of the Michaelis–Menten type as the systemic concentration L_t approaches L_{max}.

$$K_{ef}(D_t) = K_{max} \cdot L_t(1 + m_0/D_t) \quad (11)$$

$$K_{ef}(T_t) = K_{max} \cdot L_t(1 + m_0/T_t) \quad (12)$$

$$K_{ef}(M_t) = K_{max} \cdot L_t(1 + m_0/M_t) \quad (13)$$

K_{ef} for every separate metastasis is increased as the corresponding residual tumor mass approaches the level of a single cell.

In order to obtain a significant immune response it is also necessary to consider the required minimum volume of isolated tumor tissue to trigger a massive activation reaction. In the absence of vascularization as in early embryonic growth before day 18 when the size of the embryo attains approximately 3 mm [39] oxygen and nutrient supply takes place by direct diffusion. By comparison, and accounting for a different metabolic rate in the early embryo which is many times higher than in an average tumor we can estimate that the minimum mass of devascularized tumor to create a stressing inhibitive pathophysiological environment should be at least 1 cm^3 or 1 g ($D_{min} \geqslant 1$ g).

Another critical factor which must not be underestimated is the immunological load concerning the total mass of devascularized tumor (D) in relation to body weight (W). If the burden of immunolysis is excessive, the immune system may not be able to cope with all potential systemic threats, especially, contingent infection and sepsis endangering the organism as a whole. Symptoms typical for a weakened immunity may appear and the normal response to ATID will be compromised.

This is explained by the circumstance that with increasing mass of the devascularized tumor a larger number of leucocytes must be mobilized to rapidly eliminate the isolated tumor. This is to safeguard the organism from infection and the development of sepsis at the site of the devascularized tumor. However, general immune defense mechanisms are also diminished because of the impaired accessibility of leucocytes to the large mass of the devascularized tumor. We also have to consider the natural limit to the rate of replenishment of decaying leucocytes in the turnover of the immune system functions which are all involved at the same time in the course of immunolysis of the tumor mass.

The critical load D/W or limit mass of devascularized tissue in relation to body weight has not been studied under extreme conditions. However, there are practical indications of safe limits when high loads have been well tolerated without any negative consequences. Here we can mention the tolerated devascularization of a 180 cm intestinal segment in the minipig model where D/W was approximately 1% of body weight [4].

A demonstration study (unpublished experiment) showed that the devascularization of the kidney and spleen together with a colon segment of 50 cm^3 in the minipig model was perfectly tolerated (unpublished experiment, performed by Dr. Fortýn and Dr. D. Kalina in the Institute of Animal Physiology and Genetics, Liběchov, Czech Republic, on the 6th of October 2000). A revision of the animal performed 13 weeks after this devascularization showed practically complete immunolysis of the organs and no trace of the isolated colon segment which was stitched to the abdominal wall. The devascularization load D/W in the given experiment was also estimated as 1% of body weight so that the value $D/W < 0.01$ may to be considered as a safe limit without complications.

Tumor killing rate has to be higher than tumor cell growth rate

$K_{max} \sim 0.005–0.007\ h^{-1}$ provides ample potential for the elimination of metastases when cellular doubling times are around 1 week which is equivalent to $G_{ef} = 0.004\ h^{-1}$ [35]. In the case of very aggressive cancers when doubling times of metastases can be less than 1 week ($G_{ef} > 0.004\ h^{-1}$), the critical circumstance is that maximum levels of immunolytic activity have to be maintained until the elimination of all metastatic loci. This requires the immune system response to be in the full activation phase. When the devascularized mass has not yet been completely immunolysed, the systemic concentration of CTL is at the maximum level, K_{ef} is practically equal to K_{max} and thus $K_{ef} > G_{ef}$ which is the condition required for metastases to be eliminated. However, when immunolytic activity declines and $K_{ef} \ll K_{max}$ the result is that $K_{ef} \ll G_{ef}$ and metastases continue to grow.

Uncompromised immune system

All patients on which ATID was successfully performed by Dr. Fortýn, most of them diagnosed with advanced cancer normally considered inoperable to obtain a curative outcome, did not receive chemotherapy before the procedure. This critical circumstance was not always respected in later applications of ATID. In an official clinical trial carried out to test the safety of ATID only the recovery of white cell counts to the limits of standard physiological levels was considered as a sufficient safeguard for ATID to be effective [20]. This assumption has been disproved by the negative results observed in all cases when patients have received chemotherapy before ATID. We now can only assume that after several cycles of chemotherapy the initially present pool of circulating leucocytes is decimated assuming white cell counts fall to less than one third of the initial value after one cycle [40]. We can expect a similar situation in the case of radiotherapy [41]. Inevitably, this concerns a large number of precursor lymphocytes and memory cells including tumor infiltrating lymphocytes (TIL) which had contact with TSA or TAA during the development of the malignancy and are in a preconditioned state with other cooperating immune system components actually awaiting further co-stimulation necessary in the activation of ECTL. TIL as functional memory cells after isolation from cancer tissue are being employed as autologous activated precursors in special immuno-therapeutic techniques [42]. The reported positive results actually indicate their possible active role in ATID. Apparently, in the absence of these pre-conditioned TIL cells the generalized immune response typical for ATID cannot occur.

Evaluation of hypotheses and conclusions

The critical physiological conditions in Table 3 define the limits of ATID and the validity of the hypotheses. The biological principles and biomolecular mechanisms of ATID not only explain the incoherent results obtained in the unsystematic application of the

Table 3
ATID – critical limiting factors.

Relationship	Physiological condition	Limit value
$K_{ef} \gg G_{ef}$	Specific systemic immunolytic killing rate vs tumor cell growth rate	–
Rd = D/T	Required devascularization ratio (determined by model simulation)	$\gg 5$
D/W	Maximum devascularization load	<0.007
D_{min}	Required minimum devascularized tumor mass	$\gg 1$ g

D: total mass of devascularized tumor, T: mass of freely growing tumor tissue remaining in body after ATID, W: body weight.

method when limiting clinical factors were either ignored or were not known but also the disappointing results of numerous immunizing techniques using vaccines which are prepared and activated *in vitro*.

The reason of the difference between reported applications of ATID which resulted in a curative outcome [5] and an official clinical trial of the technique which has not shown any significant therapeutic effect [20] is exemplified by a special case report described hereunder [19], which reflects the very limits of the criteria in Table 3.

Special case of ATID intervention

A 41 year old man underwent in August 2000 an explorative laparotomy and was diagnosed with an adenocarcinoma in the rectum and sigmoideum with metastases in liver and para-aortal nodes [19]. In view of the advanced stage of the cancer a resecting intervention was abandoned. Chemotherapy had to be stopped after the first dose, because the patient could not bear the therapy, and refused further treatment. Five months later as a last resort in a state of visible cachexia the patient volunteered to undergo ATID, which was performed by František Chaloupka, associated professor.

On the 23rd of January 2001 the tumor was devascularized and a stoma terminalis of the sigmoideum is made. After the operation the patient fully recovered without any serious complications. The devascularized tumor was resorbed and follow-up sonography and CT examinations carried out over a period of two years showed complete elimination of the liver metastases. No new development of cancer was detected. Nearly three years after the ATID intervention it was reported that the patient succumbed suddenly to myocardial infarction at his home where he lived alone.

From reported results and personal communications in the case of patients who had already undergone standard therapy, namely chemotherapy or radiotherapy or both, the application of ATID was largely unsuccessful. Whenever there were some initial signs of an immune response and an apparent stabilization in the progression of the disease, these effects were very short lasting and the development of the cancer continued. The possible irreversibility of the damage caused to the immune system in its integrity by chemotherapy may be manifested very individually and even a longer recovery period may not be sufficient to change the unfavorable immunological status of the patient. In the described case chemotherapy was abandoned already after the first dose. We may therefore assume, that in this particular case, the deleterious effect of chemotherapy on the expected immune response to ATID was only minor and has not compromised the favorable development actually observed following the intervention.

Size of tumor: The mass of the devascularized tumor in this case is estimated at $D = 300$ g, the weight of patient $W = 50\ 000$ g and total non-devascularized cancerous mass, mainly metastases remaining in body $T = \sim$30-60 g. This gives the following critical coefficient values: $D/W = 0.006$ and an estimated Rd = $D/T > 5$–10. Here we can see that the surgical intervention was actually performed within the critical limits indicated in Table 3.

Response time: The main period of immunolysis of the devascularized primary tumor in this case is estimated to have exceeded two months, according to CT-scans and model simulation, thus giving a sufficient level of sustained systemic ECTL activity to eradicate the liver metastases in about the same lapse of time.

Significance of hypotheses: The first important observation in the described case is the progressive elimination of liver metastases accompanying the resorption of the devascularized primary tumor following ATID in an advanced stage of the disease. The second was the absence of any serious adverse reactions to the total devascularization of a larger mass of the tumor (about 300 g) left *in situ* in the body. We may therefore conclude that a curative outcome can be expected if ATID is applied under the conditions pointed out in the hypotheses. Thus, while the method may be applied safely and effectively in many current clinical cases involving different types of cancer provided we meet the critical limiting conditions of the presented hypothesis, there are situations when this may not be directly possible.

Special cases: It may occur, especially in some very advanced stages of the disease that the mass of primary tumor to be devascularized represents a burden too heavy for the immune system to cope with. In such a case when $D/W > 0.01$, partial ablation of a tumor is possible prior to ATID. A special situation also arises when the mass of primary tumor to be devascularized is not sufficient to meet the required Rd value. In such cases it is possible to devascularize additional tumor mass, such as invaded lymph nodes or other metastases, in a repeated ATID intervention when immunolytic activity declines. It is also possible to perform a partial standard resection, apply ATID on the remaining mass of primary tumor and conserve the ablated tumor using cryogenic procedures with cryoprotective agents to preserve immunogenicity and use it for repeated immunization. This can be performed by employing a medical device which allows safe only little invasive repeated immunization following the ATID principle [43]. Resected appropriately defrosted tumor to preserve immunogenicity is enclosed in a capsule allowing the passage of immune system factors and lymphocytes across a selective microporous membrane in both directions while cancer cells remain trapped inside. The device can also be used directly for provoking a strong immune response with biopsy samples of soft tissues that cannot be devascularized *in situ* as in cerebral cancer. Here we can also foresee the use of the device for repeated immunization interventions in laparoscopic surgery application of the ATID principle.

Finally, it is necessary to mention the exceptional situation when the difference $K_{ef} - G_{ef}$ for metastases is either too small or possibly even negative. In such cases we can also envisage the use of the microporous membrane device in combination with methods that can either potentiate the immunolytic activity elicited by ATID or slow down the growth of cancer cells. In the first place this opens a wide field for the application of specific immunomodulating cytokines stimulating the generation and activation of CTL but also agents capable of blocking oncogenic pathways such as STAT3 to inhibit tumor growth [2]. Secondly, we may consider the application of hormone antagonist therapy in hormone dependent cancers [44] or proteolytic enzyme cancer therapy which relies on the selective effect of pancreatic proenzymes in the inhibition of tumor cell growth [45].

Conclusions

ATID might be a viable option in the treatment of solid cancers with or without metastases. The therapeutic effect of ATID should be thoroughly explored in clinical studies with special emphasis on the physiological criteria described in this article. The clinical

inclusion and exclusion criteria for volunteering potential patients should be defined accordingly to assure maximum therapeutic benefit following the application of ATID.

Conflict of interests

The authors report no conflict of interests.

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