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Review

Cryoimmunology: Opportunities and challenges in biomedical science and practice

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ABSTRACT

Autologous and allogeneic cryoimmunological medicine is a brand new branch of biomedical science and clinical practice that examines the features and formation of the immune response to immunogenic properties of normal and malignant biological structures altered by ultralow temperature, as well as specific changes in the structural and functional characteristics of immune cells and tissues after cryopreservation. Cryogenic protein denaturation phenomenon provides important insights into the mechanisms underlying the damage to cryogenic lesions immediately after freeze–thawing sessions in bioscience and medicine applications. The newly formed cryocoagulated protein components (cryomodified protein components) are crucial in cryoimmunology from the perspective of the formation of immunological substances at ultralow temperatures. Dendritic cells and cryocell detritus (cryocell debris) formed in living biological tissue after exposure to ultralow temperature *in vivo* may be an indication of one of the essential mechanisms involved in the cryoimmunological response of living structures to the impact of ultralow temperature exposure. Hence, the formation of new autologous and allogeneic cryoinduced immunogenic substances is a novel concept in biomedical research globally. Accordingly, this review focuses on issues concerning the peculiarities of the interaction of the immune system with a dominant malignant neoplasm tissue after exposure to subzero temperatures, considering the original cryogenic technical approaches. We present an overview of the state-of-the-art methods of cryoimmunology, and their major developments, past and present. The need for the delineation of structural and functional characteristics of the biological substrates of the immune system after cryopreservation that can be used in adoptive cell therapy, especially in cancer patients, is emphasized.

1. Introduction

Analysis of public health statistical data from the past decades has confirmed the rise in mortality rates of cancer and related diseases despite the excessively high financial costs associated with diagnosing and treating these diseases. Immunotherapeutical approaches, including cryoimmunology and anticancer vaccines, are being increasingly used to improve the quality of life of senior citizens and to treat associated diseases in such patients, particularly those with various forms of cancer [18,33,46,84,96].

Cryoablation is used as a treatment for cancer. It involves killing cancer cells by exposing them to ultralow temperatures. At the end of the 1960s, Shulman et al. [113] reported the production of specific antibodies against cryoablated tissue in rabbit. The formation of antibodies in response to cryosurgery may well be caused by nonspecific damage associated with freezing and thawing, which results in the release of massive amounts of ordinarily inaccessible antigens from the rabbit accessory tissue. The necessity of utilizing antigen–adjuvant combinations for the experimental production of autoantibodies has now been replaced by another approach. In a way, alterations in tissue structure and/or tissue chemistry upon exposure to ultralow temperature behave in a manner similar to an adjuvant. This "cryoadjuvant" effect can lead to the production of autoantibodies. The mechanism involved is not yet clear, but studies are in progress. It has been proposed that this general method of eliciting autoantibodies and investigating the nature of the response be designated "cryoimmunology" [135].

The development of techniques involving the generation of new

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Abbreviations

AACM	autologous and allogeneic cryoimmunological medicine
ALDH	aldehyde dehydrogenase
APC	antigen-presenting cell
BCI	biocryoimmunology
BCIAb	biocryoimmune antibody
BCV*i*	individual biocryovaccine
CA	cryoinduced antigen
CAI	cryoanticancer immunology
CAV	cryoanticancer vaccination
CCD	cryocell detritus or cryocell debris
CI	cryoinduced immunogen
CIS	cryoinduced immunogenic substance
CPC	cryocoagulated protein component
CPD-phenomenon	cryogenic protein denaturation phenomenon
DC	dendritic cell
DMSO	dimethyl sulfoxide
FPC	fetal progenitor cell
ICC	immunocompetent cell
NK	natural killer
NKG2D	natural killer group 2D
NP	nanoparticle
NT	natural tolerance
PD-L1	programmed death ligand-1
TIL	tumor-infiltrating lymphocyte
Treg	regulatory T cell

cryoinduced immunogenic substances upon exposure to ultralow temperature is a novel concept in current biomedical science and practical experience, and includes autologous and allogeneic cryoimmunological medicine, also encompassing cryoanticancer immunology [75–77]. Based on the studies mentioned above, there is now every reason to discuss the need of introducing this "new" term into classical immunology as a science.

"Cryoinduced antigen" is one of the main terms of autologous and allogeneic cryoimmunological medicine (AACM), and reflects new immunogenic characteristics and properties of cryopreserved immune cells and tissues, e.g., in the form of various types of immunocompetent cells (ICCs) and antibodies. The intensification of this direction of research may lead to the formation of new disciplines, such as cryopharmacology, in the near future, heralding the advent of autologous and allogeneic cryovaccine production technologies (including cryoanticancer technologies) obtained by the intended and dosed variation of cryoinduced immunogenic substances (CIS) in cryoeffect modes. This will comprise a qualitative leap in improving the effectiveness of immunotherapeutic approaches in medicine, particularly in oncology.

The mechanisms of the response of the immune system and its substrate to cold, including ultralow temperatures, are unclear [43,76,77,140], and the pathogenetic and physiological responses of biological objects, including primary malignant tumors and autonomous malignant micro- and macrosystems, to the effects of ultralow temperatures have been only partially explained [54,72,86]. Therefore, characterization of the living matter's responses to ultralow temperature and delineation of the formation of interactions in the neuroimmune block as a combination of systems responsible for the homeostatic equilibrium in the body are fundamental tasks of bioscience, aimed to optimize the existing approaches for the prevention and treatment of various diseases. This branch of research is gaining special significance as part of the recently announced and developing discipline of autologous and allogeneic cryoimmunological medicine, proposed and applied for the benefit of mankind [34,38,73,75–77,79,80,85,87].

In the current review, we provide an overview of cryoimmunology. We discuss a discipline of biocryoimmunology (BCI). We also present some major challenges and opportunities in the field, emphasizing the formation of immunological substances at ultralow temperatures.

2. Key concepts in cryoimmunology

2.1. Cryoimmunology

The term "cryoimmunology" was first introduced in a report Shulman et al. [113]. Coining this term, the authors referred to the science of studying the phenomena associated with the emergence of a product of the immune system, a predominant antibody, as a response to the introduction of frozen tissues from male rabbit from the same line, i.e., isoimmunization. Further studies are in progress to evaluate the molecular size of the predominant antibody at various stages after freezing, and to examine the outcome of combining isoimmunization by injection with freezing. Other tissues as freezing immunogens are also tested. At present, it seems appropriate to use the term "cryoimmunology" to designate these phenomena and related studies.

The cryoimmunization response demonstrated experimentally can be comparable, to a certain extent, with the development of autoimmune responses to self-antigens that had been altered under certain circumstances. Furthermore, the immune-inducing effect of freezing can be compared with that of adjuvant during its combined application with a specific antigen. One of the mechanisms of adjuvant activity is antigen absorption onto the adjuvant, which results in antigen enlargement, with a subsequently improved capture and active presentation of the antigen by phagocytosing cells [5]. Cryodenaturation in some cases promotes the formation of molecular conglomerates, leading to the enlargement of the antigenic substance [82,83]. CD38 and CD95 molecules are examples of tumor antigens in breast cancer [100].

2.2. Cryoimmunotherapy

Soanes et al. [115,117] observed a decrease in metastatic loading in patients with prostate cancer whose primary tumors have been cryoablated. Two patients with carcinoma of the prostate were treated by cryosurgical destruction of the primary tumor; a regression of metastases was then observed. The implications of this "immunocryothermic response" are discussed. Of note, the scientists who introduced the term "cryoimmunotherapy" also observed a strengthened immune response after repeated cryoablations. The data from a coauthor of this research group published 2 years later were considered in the same context [1].

2.3. Immune response to freezing biological structure

Understanding the immune response against structures that are newly formed in response to freezing stems from the basic principle of the function of the immune system in mammals, i.e., the formation of natural tolerance (NT), that is, unresponsiveness to self-antigens [10,13,46,69]. NT develops to all immunogenic epitopes of proteins of an organism (excluding sequestered antigens). The development of NT is complicated, and involves the creation of a specific ICC profile in the central and peripheral organs of the immune system. Impaired NT to self-antigens leads to the development of autoimmune reactions that manifest as disease [49]. The development of an immune reaction to self-antigens may also be an outcome of other mechanisms. The change in protein structure in response to any physicochemical factor leads to the appearance (expansion) of immunogenic epitopes that had been "hidden from view" from the own immune system in the physiological state. In fact, redifferentiation of epitope diversity, called "epitope

spreading" in immunotherapy, takes place in tissue as a result of exposure to low temperature, including in tumor tissue [59,128].

2.4. Peptide structure at ultralow temperatures

The observed cryogenic protein denaturation phenomenon (CPD-phenomenon) encompasses the original reports on the response of a living organism to ultralow temperatures [79,82,83,86,87]. The discovered CPD-phenomenon, during which the cryocoagulated protein components—cryomodified protein components—are located in the mitochondrion, is one of the main early and late mechanisms of cryosurgical injury studied *in vivo* using transmission electron microscopy (Fig. 1).

The CPD-phenomenon provides important insights into the mechanisms of cryogenic lesion damage immediately after a freeze–thawing session in biomedical science. In fact, the newly formed cryocoagulated protein components—cryomodified protein components—are one of the crucial links in cryoimmunology, and autologous and allogeneic cryoimmunological medicine, i.e., the immunological substances formed at ultralow temperatures [78–80,82,83,85]. A localized deep-freezing of living matter, including malignant tumor cells and tissues, can stimulate the immune system, and trigger a multifarious cascade that destabilizes its structural and substantial components, primarily locally, that could then spread to a systemic level.

Regarding the changes in peptide structure upon exposure to low temperatures, accompanied by the phenomenon of epitope spreading, one should always expect changes in immunogenicity. This has been confirmed by previous studies [70,86,87]. For instance, cord blood cells exert an allostimulating effect in a mixed lymphocyte culture after cryopreservation at a rate of 1 °C/min. Further, following cryopreservation, no increase in the expression of HLA-DR antigens is observed in these cells. In another study, a strong inhibition of B16 melanoma growth and long-term survival was observed in mice immunized with a cryolysate of cells from this line [7]. The authors hypothesized that the treatment increases the immunogenicity of tumor antigens and thereby induces immune reactions leading to tumor rejection. In contrast to the administration of a melanoma cryolysate, immunization of mice with frozen cells or a "raw" butanol extract only resulted in tumor growth inhibition without prolonging the animal life expectancy. This observation emphasizes the notion that freezing itself alters not only the immunogenic characteristics of tumor tissue but also affects the features of the biological object's cryopreservation implementation. Different molecular-level changes in the structural and functional characteristics of tumor cells (including cancer stem cells) depending on the freeze–thaw regimen have been recently demonstrated [41,42].

Regardless of intense study, the characteristics of the structural changes in proteins after exposure to ultralow temperatures remain vague [48]. Electron microscopy and cryoelectron microscopy with vitrification can be used to address this question [for reviews, see 82, 83].

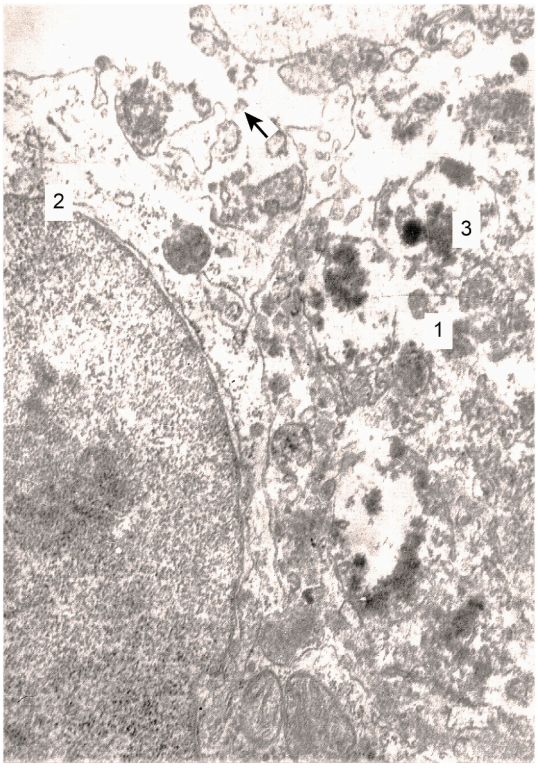

Fig. 1. *In vivo* changes in the liver tissue 24 h after cryosurgery at –180 °C. Electron micrograph of liver tissue in the cryogenic center zone, with hepatic cells (1) and macrophages (2) indicated. The plasma membrane of the hepatic cell (arrow) is completely lysed. The coagulated cryo-protein components—cryomodified protein components (3)—are situated in the mitochondria. Magnification, × 20,000 (Korpan, unpublished).

3. Biocryoimmunology (BCI)

3.1. Overview

The term "biocryoimmunology", namely, autologous and allogeneic cryoimmunological medicine, and its definition were first introduced by Korpan [76,77]. The BCI field of study includes investigations into the specific changes in the structural and functional characteristics of the biological substrates of the immune system upon cryoimpact and the biological cryogenic response. It also encompasses the formation of new immunogenic biological substances of an autologous and allogeneic nature as an effect of exposure to ultralow temperatures, achieved by active rapid freezing, and simple passive and slow intracorporeal warming *in vivo* and extracorporeal warming *in vitro* (Fig. 2A–C). The sequence shown in Fig. 2 reflects the generation of aseptic autologous cryocell detritus that can be used for cryoimmunological medicine in cancer patients to activate a specific cellular and humoral cryoanticancer immunological response cascade [73,74]. Complete cryofragmentation of cancer cells, yielding novel biocryocancer antigens, is achieved via an extracorporeal active–passive ultralow freezing–thawing cycle with a formation of new cryoinduced protein particles that have new immunological characteristics and new properties of cryostimulated immune system [75,76]. Individual biocryovaccines (BCV*i*), including hematological substances, are new biological cryoantigens generated from autogenous biological material within an individual, in an intra- or extracorporeal manner. This includes patients with malignancies, and bacterial or viral diseases, and such material as the blood, plasma, lymph, urine, ascites fluid, pleural effusion, amniotic fluid, and other living matter, that is altered by ultralow temperature upon active freezing and passive thawing [77,83].

Based on currently available scientific data [73–75], it can therefore be argued that autologous and allogeneic cryoimmunological medicine (AACM) reflects the immune response to the immunogenic properties of normal and malignant biostructures in a body that had been affected by exposure to ultralow temperatures, and cryoinduced immunogenic substrates in human tissues (e.g., cryocell mini-particles). Cryocell mini-particles, which induce the production of human biocryoimmune antibodies (BCIAbs) and can interact with them, trigger a cascade of responses associated with the manifestation of a specific immune response, i.e., the cryoimmune response. Further, after cryoablation, malignant tissues, such as a solid tumors, and human micrometastases and macrometastases, can potentially act as biological inducers of autologous and allogeneic cryoanticancer immunity, which is an important element of anticancer defense. They are therefore termed biological anticancer defense inductors. Such autologous cancer cryocell miniparticles are actually cryoinduced cancer antigens, which trigger a

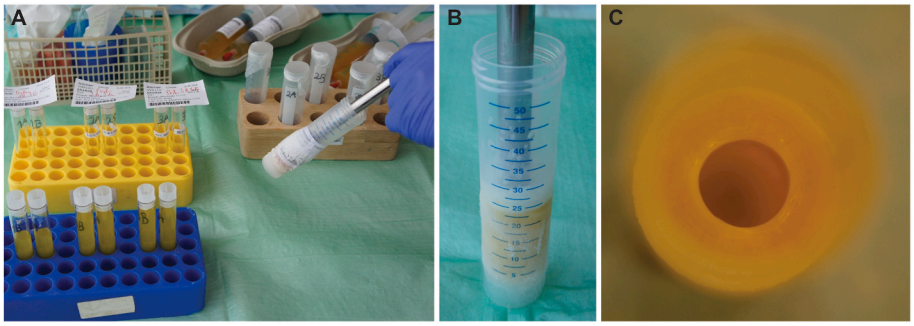

Fig. 2. Development of cryoinduced immunogenic substances—cryoinduced immunogens—for a patient with an advanced-stage pancreatic adenocarcinoma. Principles of generating of a novel individual autologous and allogeneic biocryovaccine using a technical standardized extracorporeal process, by applying ultralow temperature, with liquid nitrogen as the cryogenic medium. (A) Deep-freezing of malignant ascites at ultralow temperature (−180 °C) *in vitro*. (B) Active and rapid extracorporeal deep-freezing of a malignant ascites fluid *in vitro*. (C) Passive and slow thawing of malignant ascites immediately after deep freezing at −180 °C *in vitro*, viewed from above (Korpan, unpublished).

cascade of immune responses. Finally, circulating biological material, e.g., in the bloodstream of the tumor carrier, and representing new immunogenic substances (cryoantigens) generated by an intracorporeal or extracorporeal cryomanipulation of a pathological tissue in patients with malignant neoplasms and background (bacterial) viral diseases can be attributed to autologous and allogeneic BCV*i*. In general, the blood, plasma, lymph, urine, ascites fluid, pleural effusion, amniotic fluid, and other biological material that is susceptible to freezing–warming according to the developed protocol for activating a specific cell and humoral immune response cascade can be classified as liquid phase immunogenic substrates [76–78].

The active principle of biocryovaccines is associated with the formation and entry of low-molecular weight compounds (protein molecules as the main element of the different levels of organization of body structures: cells, tissues, and organs), identified by the immune system as alien substances, into the body's fluids. These particles act as antigens and initiate multiple immune events, first locally and then systemically throughout the body. Activation or initiation of the biocryocancer antigen immune response can be compared with the bioimmune bomb effect, which includes the standard combination of the main phenomena of the immune response cascade, namely, coupling of amplified principal cascade phenomena. These phenomena act as "bioswitches", and activate or stimulate cellular and humoral antitumor immune defense factors [35,44,75–77].

3.2. Cryopreservation coupled with BCI

Expanding the application field for ultralow temperatures in medicine has highlighted the need for cryopreservation of the widest range of ICCs in the form of a suspension or tissue substrate [57,58,63,109,127]. According to recent studies, ultralow temperatures are an extremely aggressive environmental factor capable of changing the state of immune system substrates at all levels of organization [30,36,124]. In this regard, a new but no less important question arises regarding the need for comprehensive attestation of the features of these changes. Further, the possibility of operating cryopreservation regimens that can selectively eliminate or enrich certain subpopulations of ICCs in a heterogeneous suspension is currently discussed [37,51,53,125]. Research findings emphasize the extremely important second task of autologous and allogeneic cryoimmunological medicine, which should be formulated as characterization of changes in the structural and functional organization of the immune and lympho-hemopoietic system components in cancer patients at the cellular, subcellular, and molecular levels and, most importantly, their modification profile after exposure to ultralow temperatures (cryopreservation).

Such a formulation of the question reminds us of the problem associated with the expansion of a tumor within the body, regardless of the location, and the necessity for improvements in antitumor therapy approaches based on the need and possibility of using substances other than biological fluids of cancer patients to obtain BCV*i*. It is logical that, while a tumor can secrete specific biomarkers into the bloodstream (and other fluids), the biomarkers primarily appear at the site of their formation. It is significant that these markers, which posses a native structure, are not recognized to any great extent by the immune antigen-presenting cells (APCs). Such biomarkers include, e.g., CA 125, CA 15-3, CA 72-4, CA 19-9, carcinoembryonic antigen, and alpha fetoprotein [28]. The antigens of dying destroyed tumor cells (i.e., antigens on the internal contents of tumor cells), which may be related to the development of immune inflammatory and autoimmune reactions in oncological diseases, also enter the bloodstream [4]. The expansion of the spectrum of biological material of malignant tissue, which can be subjected to destruction by low temperature, increases the likelihood of a specific immune response to them, with the ensuing growing recognition and elimination of tumor structures by the immune system. All types of immune protection mechanisms, including dendritic cells (DCs), can be involved in this type of antitumor effect [62]. Since tumor-infiltrating lymphocytes located in different areas of the tumor are also affected by low temperature, we suggest a possible transformation of their antitumor potential upon exposure to low temperature. As previously reported [15], the expression of the programmed cell death 1 (PD-1) receptor (CD279) and its ligand, programmed death ligand-1 (PD-L1), on the surface of lymphocytes is reduced after cryopreservation. The PD-1 receptor is mainly expressed by activated T cells ($CD4^+$, $CD8^+$), as well as natural killer (NK) cells, B cells, and tumor-infiltrating lymphocytes (TILs). It inhibits T-cell activation by interacting with PD-L1 (B7–H1) and PD-L2 (B7-DC), which, in turn, are expressed by the tumor, stroma, and circulating immune complexes [67].

In 2018, Ellison and Khondze discovered the "control point" or "checkpoint" molecules of the immune response that can stop the development of an immune response in malignant neoplasms [126]. In light of this, we suggest the possibility that cryoexposure intensifies the antitumor properties of TILs. It is also possible that upon cryoablation, the ligands expressed on tumor cells at checkpoint will acquire immunogenicity and act as targets for the immune cells, with antitumor potential.

The ligands of the NK group 2D (NKG2D) receptor, the expression of which is associated with cell stress, proliferation, and oncogenic mutations, play slightly different roles during tumor growth. For example, in leukemia, unlike in other tumors, cancer stem cells do not express NKG2D-L, which allows them to elude immunological surveillance [103]. Since cryoablation is a powerful cellular stress factor, it is likely that NKG2D-L will be expressed in the cancer stem cells that remain after cryosurgery, although not all solid tumors are governed by this molecule's patterns of expression [14].

4. Cryoeffect and cytokines

In the context of the possibility of using cryostressed biological fluids and other material as vaccines, the probability of the development of inflammatory reactions is a cause for concern. According to some researchers, an antigenic load in this form can initiate the production of proinflammatory cytokines and, consequently, lead to multi-organ inflammation [133]. It is likely that the volume of cryoablated material plays a specific role in the development of events in this direction.

For example, development of systemic inflammation in experimental animals that underwent liver cryoablation in a volume of 30% has been shown [133]. An aggravating factor in this case may be inflammation chronicity, which also induces the development of autoimmune diseases [50]. In this regard, the development of a cryoablation-based approach to cancer treatment requires a thorough preliminary evaluation under specific experimental conditions. The use of tumor cells destroyed by cryoablation as cryovaccines may indeed potentially lead to other complications. Based on some studies, effective immune response to tumor antigens from destroyed cancer cells may be accompanied by immune cross-responses to healthy tissue antigens because of epitope spreading [101,129]. Nevertheless, such a phenomenon has not yet been reported after tumor cryoablation.

5. Considerations of cryoablation and stimulation of the immune response in cancer

5.1. Cancer stem cells and the cryoeffect

Since the publication of a study by Yoon et al. [136], in which the possibility of isolating circulating tumor cells, including cancer stem cells, from the peripheral blood was described, the potential of using vaccines based on these cells destroyed upon exposure to low temperature has gained prominence. In this context, attention should be paid to research pointing to the presence of cancer stem cells in solid tumors in the bone marrow and in positive sentinel lymph nodes [6,99,108]. The recent division of cancer stem cells into those in initiating and metastatic tumors, which have different properties [31], emphasizes the relevance of this kind of research. In this case, preliminary research is crucial, although it might be difficult to evaluate the effectiveness of such vaccines in experimental models. Nevertheless, even now, it is possible to test both, the diversity of cancer stem cells and their cryolability (cryostability) in experimental models [40,45,55,56,88].

A tumor is a heterogeneous hierarchical structure containing cells with varying degrees of differentiation. When constructing a conditional hierarchical ladder of the functional potential of tumor cells, it is generally accepted that cancer stem cells have the highest oncogenic capability, the phenotypic features of which depend on tumor histogenesis and location. However, some markers (e.g., CD133 and CD44) are common to cancer stem cells in many tumor types. Further, high expression of the CD44 molecule ($CD44^{high}$) is characteristic of cancer stem cells in a number of tumors. These cells are characterized by an individual antigenic spectrum that distinguishes them from other tumor cells [60,94]. A previous study demonstrated significant differences in the antigenicity and/or immunogenicity of cells with a high level of aldehyde dehydrogenase (ALDH) ($ALDH^{high}$) and a low level of ALDH ($ALDH^{low}$) isolated from tumor specimens from patients with head and neck squamous cell carcinoma, indicating the existence of unique cancer stem cell antigens in the $ALDH^{high}$ population [106]. This finding is not surprising if we consider that ALDH is a protein molecule, the quantitative content of which determines immunogenicity even at the cancer stem cell membrane.

5.2. Cancer stem cells and vaccines

Vaccines against cancer stem cells have been successfully used in recent studies to malignant glioma [68,139]. It can be assumed that the use of anticancer cryovaccines containing cancer stem cell proteins (glycoproteins) will lead to their eradication through the activation of a specific cell and humoral immune response to certain immunogenic sites on these cells and, therefore, to an increased probability of curing cancer. Studies demonstrating the complete disappearance of metastatic nodes after cryosurgery performed in primary tumors indirectly support this notion [3,116,117].

A retrospective analysis of the above results, as well as the observations of prostatic carcinoma described in Refs. [2,32], is of particular interest from the point of view that the findings indicate a less pronounced therapeutic effect of cryosurgery during operations on late-stage tumors than on early-stage. In one study, the authors noted lower blood levels of immunoglobulins and tumor-associated carcinoembryonic antigen characteristic of cancer stem cells in patients with stage 4 tumor growth than in patients with stage 3 [21]. Data obtained almost 50 years ago concur with more modern and unique experimental data acquired using the *in vivo* experimental model of Ehrlich carcinoma [47,55]. Specifically, diametrically opposite effects of cryoexposure on the survival of cancer stem cells in this tumor in the early and late growth stages were reported [55]. In other words, the effect of cryoeradication of cancer stem cells in "young" tumors is significantly higher than that in "old" tumors, i.e., those with a long developmental period. Furthermore, the cryoeffect had a renewal effect on cancer stem cells in tumors with long developmental period (old). Varying resistance to the cryoeffect and different levels of differentiation in the "stem cell continuum" in tumor tissue cells were also noted. This supports the relevance and effectiveness of cryoeradication on the malignant process at the early stages of tumor development.

Although several cases of spontaneous remission of metastases after cryoablation were reported in earlier studies [117], further investigations in clinical practice and in experimental models have revealed weak or absent immune responses after this procedure, despite the massive release of proteins upon tumor cell death [25,110,131]. Without going into details of the tumor development features in human and in experimental animals, or the immune response to these structures, the observation that the immunogenic properties of tumor proteins are mandatory for cancer therapy is obvious. It has therefore been suggested that the immune response can be enhanced by combining cryoablation with immunotherapy that targets APCs or modulates T-cell function.

5.3. Cryoablation, DCs, and cryocell detritus

Following on from the point made in section 5.2, recent studies have proved the validity of the combined use of cryoablation and the introduction of autologous DCs obtained from bone marrow mononuclear cells or the peripheral blood after a preliminary corresponding *in vitro* treatment [24].

To acquire antitumor activity, mature DCs are loaded with protein or peptides [23,24]. The role of DCs in antitumor immunity has been empirically confirmed by Ralph Steinman, who discovered these cells in 1973 [119]. Having suffered from cancer throughout virtually the first decade of the 3rd millennium, Steinman used autologous DCs grown *in vitro* to combat this ailment. While it is difficult to convince skeptics of the viability of the antitumor "autotherapy" described by Steinman, he has convinced the scientific world that DCs play a key role in the formation of acquired immunity, including antitumor immunity, in his recent scientific studies [118,120,121]. This finding is supported by other similar reports [91].

Specific cell debris, the cryocell detritus or cryocell debris (CCD), was discovered and observed *in vivo* in early 2000s by Korpan, using a cryosurgical approach [82,83,86,87]. Specifically, a cryoprobe within the temperature range of −80 °C and −180 °C was placed on the parenchyma of the pancreas and liver tissue of an animal. Electron microscopy of the animal's pancreas and liver showed cell detritus and fibrin fibers 24 h after cryosurgical intervention at −180 °C, i.e., after a single freeze–thaw cycle (Fig. 3).

In this context, the observed changes that occurred after exposure to ultralow temperatures of a living biological tissue *in vivo* could be an indication of one of the important mechanisms underlying the cryoimmunological response of living structures to ultralow temperature (Fig. 4).

Furthermore, the CCD, formed as a result of the cryoimpact or cryoablation of tumor malignant cells and tissue, constitutes an effective antigen depot for the induction of therapeutic antitumor immunity

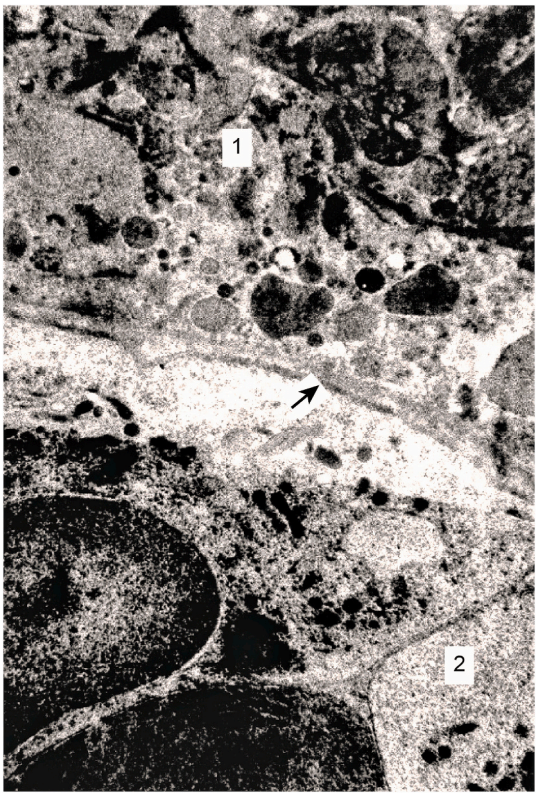

Fig. 3. Electron micrograph of ultrastructural changes in pancreatic tissue following exposure to ultralow temperature (–180 °C) *in vivo*. Cell detritus (1), blood cell (2), and cell membrane (arrow) are indicated. Magnification, × 18,000.

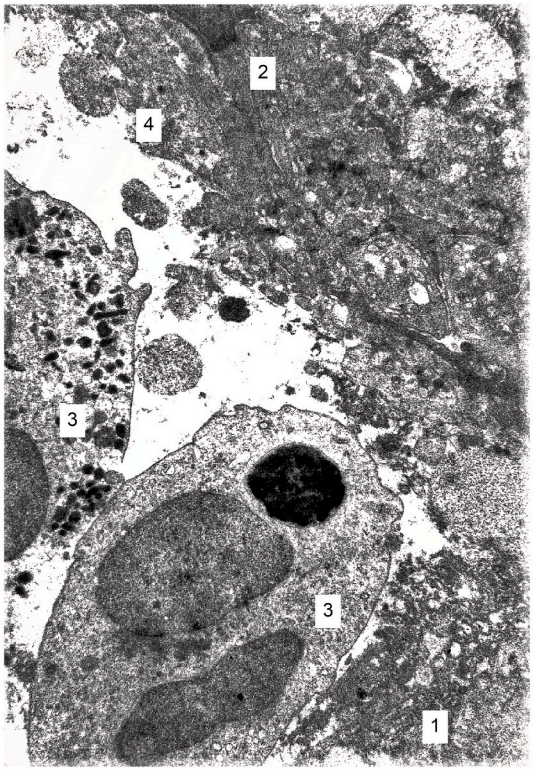

Fig. 4. Electron micrograph of ultrastructural changes following exposure to ultralow temperature (–180 °C) of liver tissue *in vivo*. Lysed hepatic cells (1), endothelial cells (2), leucocytes (3), and cryocell detritus (clearly apparent) (4) are indicated. Magnification, × 12,000.

mediated by CCD [74,77,81,85–87].

5.4. Cryoablation and the checkpoint inhibitors

During cryoablation, special attention should be paid to the "turning off" of the immunosuppressive mechanisms of tumor cells, for example, turning off the immune checkpoint blockade, such as the introduction of a mABb against CTLA4 [131]. Elimination of regulatory T cells (Tregs) and myeloid suppressor cells is also effective. Effector cells, such as NK cells [123], and certain cytokines that can enhance the vaccine-like effect of cryoablation are often introduced during cryoablation. When combining cryoablation and the injection of Toll-like receptor agonists, a synergistic effect aimed at rejecting the tumor was demonstrated, which was also associated with the activation of APCs [23,107]. Further, combined cryoablation and the administration of autologous DCs obtained from peripheral blood mononuclear cells pre-cultured with cytokine-induced killer cells has improved the overall survival of patients with pancreatic cancer in one study [98].

6. Cryopreservation: cryoprotectants and impacting factors

6.1. Factors affecting cryopreservation of cells

Factors that influence the preservation of cells after cryopreservation, which is a multistage technological process, are the type and concentration of cryoprotectant, the rate of freezing and warming of biological objects, etc. However, it is impossible to propose any specific method of freezing and warming as optimal for a wide range of cells, considering their different responses to the low-temperature preservation factors [49,51,52,93,102]. Rather, one has to agree that "one type of cell matches one cryopreservation mode" [39].

The choice of cryoprotectant for the cryopreservation of cells, namely, ICCs, is very relevant. Dimethyl sulfoxide (DMSO) is predominantly used in cryobiological studies because of its proven cryoprotective effect on various biological objects [9,12,46,49,54,130]. The first attempt at using DMSO to prevent freezer damage to living cells was reported by Lovelock and Bishop in 1959 [92]. Since then, DMSO has become the most widely used cryoprotective agent for freezing both cells and tissues. The cryoprotective properties of DMSO are based on its ability to easily penetrate the cell membrane both, to inhibit the formation of intracellular ice and to prevent injury to cells caused by severe dehydration, since extracellular ice causes water drainage from the intracellular medium. However, in clinical practice, the use of DMSO is, in some cases, accompanied by undesirable effects, such as a toxic effect on the cardiovascular, respiratory, and urinary systems, with the need for its removal before hematopoietic stem cell products administration [66,95].

6.2. Metformin, a cryoprotector with a dualistic activity

Metformin is an intriguing molecule that can be used as a cryoprotectant when freezing cells that are candidates for adoptive immunotherapy, including antitumor cells. The available studies indicate its effectiveness for freezing spermatozoa and pancreatic cells [8,17,20,97]. Its cryoprotective effect is attributed to its antioxidant properties. In addition, the cryoprotective effect of metformin can be indirectly implemented by inhibiting cell metabolism [65]. This is fully consistent with the assumption that the exogenous and endogenous inhibition of cell metabolism can protect the cell against the damaging events that occur during cryopreservation [105].

Metformin's dualistic activity is well recognized and in addition to its cryoprotective properties, it is being used in oncology as an antitumor drug, mainly directed against cancer stem cells [29]. Furthermore, there is evidence that metformin exhibits an immunotropic effect in

oncopathology [16,22], inducing an antitumor effect indirectly via the immune system. In fact, as shown in one study, its effect was completely abolished in mouse with T-cell deficiency and after *in vivo* depletion of $CD8^+$ T cells by specific monoclonal antibodies [27]. In addition, metformin induces the production of peripheral blood $CD8^+$ cells by inflammatory cytokines, such as interferon γ, tumor necrosis factor α, and interleukin 2 [132], which participate in antitumor defense [71]. Metformin indirectly mediates the increase in the number of TILs via the 5′-adenosine monophosphate–activated protein kinase signaling pathway, enhancing their functional activity [27]. Further, on one study, metformin administration to mouse with melanoma was accompanied by a decrease in the number of $Gr\text{-}1^+$ $CD11b^+$ cells, which belong to myeloid-derived suppressor cells, inhibiting, as already mentioned, the antitumor immune response in the spleen [104]. Kunisada et al. [90] reported that metformin leads to a decrease in Treg levels, in particular, terminally differentiated $KLRG1^+$ $CD103^+$ cells, i.e., active effector Tregs [114]. Furthermore, metformin induces reprogramming of the metabolic state of naive T lymphocytes towards glycolysis and activates mechanistic target of rapamycin complex 1 (mTORC1), which reduces their expression of forkhead box P3 (Foxp3) and, consequently, their differentiation into inducible Tregs. This has been confirmed in *in vitro* studies showing that the pretreatment of naive $CD4^+$ $CD25^+$ cells with metformin blocks their differentiation into tumor growth factor β-inducible Tregs because of Foxp3 inhibition [90]. Since a clear correlation exists between an increase in the Treg ($CD4^+$ $CD25^+$) levels and tumor aggravation [19,52,112], a decrease in their levels may be one of the possible mechanisms underpinning the antitumor effect of metformin. Further, the findings of Cha et al. [16] expanded the functional potential spectrum of metformin, highlighting its ability to reduce the stability and localization of PD-L1 on tumor cell membrane, and the ability to enhance the anticancer activity of cytotoxic T lymphocytes.

Of note, metformin exerts an anti-inflammatory effect [26] that can and should be considered in the context of the possible activation of the inflammatory process by cryoimmune vaccines. In this regard, the results of a number of studies that use metformin for the treatment of autoimmune diseases are indeed exciting [111,122]. This emphasizes, first, the versatility of metformin activity bordering on diametrically opposite effects, namely, from the inhibition of Treg function, which is necessary for cancer treatment, to minimizing the development of autoimmune diseases, where it is difficult to expect positive results in the absence of Treg activation. Collectively, these observations indicate the dependence of the activity of this unique molecule on many factors, namely, its dose and the initial state of the immune system, which must be taken into account when applying it in clinical practice.

7. Autologous and allogeneic biovaccines

7.1. Applications of biocryovaccines

An autologous biovaccine is generated in the course of a technically standardized process by applying cryogenic temperatures and creating a cryogenic environment [73,75–78,81,134]. Representing a revolutionary innovation in the development of anticancer vaccines, this method ensures the development of continuous physiological immune cascade responses at local and systemic levels for antitumor protection by creating cryogenic pressure in mammals, including human. The discovery of cryocell detritus (cryocell debris) and cryocoagulated protein components as well as the direction of autologous and allogeneic cryoimmunological medicine (AACM) driven by the exploitation of biovaccines suggest their leading role in bioscience, medicine, veterinary medicine, and economics.

Internal pilot studies in patients suffering from pancreatic head cancer, multiple liver metastases, and breast cancer have demonstrated the onset of a long-term antitumor immune response as a consequence of using anticancer vaccines. Furthermore, combined with biocryovaccines, chemotherapy becomes much more effective in terms of synergy. This therapeutical approach minimizes side effects [61,64,76,78,89].

7.2. Biocryopharmacology

As a product of BCI, biocryovaccines constitute a completely new principle for the production of pharmacological products and represent a quantum leap towards increasing the effectiveness of antitumor immune therapy. Based on the available data, biocryovaccines can selectively activate the immune system, leading to antitumor protection. The emerging biocryopharmaceutical industry will likely include the following new drugs: individual autologous biocryovaccines; standardized allogeneic biocryovaccines; biocryomedications, such as biochips, skin patches, powders, tablets, capsules, drops, sprays, and vials; and biocryonanoproducts. These new elements of the general approach to anticancer treatment are, in fact, derivatives of cryo-affected entities, the tangible demand for which is evidenced by the growing volume of related scientific works globally [73,74,76,78–81,85,134].

8. Perspectives

Immunotherapy is currently evolving into a major therapeutic option for cancer patients. Its clinical advances also promote massive interest in the search for novel immunotherapy targets and understanding the mechanism of action of currently used drugs. It is projected that a series of novel immunotherapy agents will be developed and assessed for their therapeutic activity in patients with malignancies. Formed in response to the appearance of BCIAbs, previously called cryoimmunization antibodies [138], these new biological entities represent immunoglobulins that are specific to antigens altered by exposure to ultralow temperature. Their formation is one of the main factors associated with the antimetastatic effect of repeated cryosurgeries [1,115,117]. Studies conducted to investigate changes in the immunogenicity of the formation of BCIAb may be a starting point for the emergence of a new discipline, “biocryopharmacology”, targeted to introduce the advances of BCI into the pharmacological industry for biocryovaccine design. This will be an entirely new, 21st century direction of research that is the consequence of the evolution of BCI and biovaccination [74,76,78,80,85].

The confirmed effectiveness of using DCs and CCD in cryoablation, and cryoimpact as an integral component of the technological process of adoptive immunotherapy drives the need to create their reserves, i.e., cryopreservation [34,59,74,77,98,118,119]. The development of cryopreservation technologies using ultralow temperatures for autologous and allogeneic CCD and DCs for clinical practice foresees the many nuances of this process, including the initial state of the bio-object before cryopreservation [34,59]. The possibility of applying the cryopreserved.

DCs and CCD used as an element of immunotherapy in the tumor process confirms the essence of BCI presented above, i.e., as “a science that studies the changes in the immune system biosubstrates’ structural and functional characteristics after cryopreservation” [82,83,86,87,98,118,119].

Furthermore, it should be noted that the additional efforts of scientists in the autologous and allogeneic cryoimmunological medicine field should not be limited to the approaches and drugs mentioned above, but should be aimed at developing new methods to expand and create fundamentally new technologies and drugs that fully incorporate nanotechnology and nanostructures [including nanoparticles (NPs)] to maximize the efficiency of homologous and allogeneic cryovaccines. A wide range of physical and chemical parameters that could be varied during the manufacturing of NPs will provide greater flexibility in therapy in terms of targeting and immunoregulatory properties. In addition, because of the ability of some types of NPs to enhance intracellular ice formation, NPs have been applied in cryoablation [64,137]. There is a general tendency to create multifunctional nanocomposites that combine diagnostic and therapeutic capabilities; for example,

vanadium derivatives (in particular, NPs and hybrid nanocomplexes based on them) are capable of solving such problems. Experiments revealed the applicability of NPs for the visualization cancer stem cells [52,55] and confirmed their ability to inhibit tumor growth [38,41,44]. Logically, combining nanostructures and low-temperature freezing factors will enhance this effect, and is a new promising area of homologous and allogeneic cryoimmunological medicine [35,40,73–76,78,81].

Applications of cryoanticancer vaccination include, but are not limited to, the prevention of cancer in individuals with hereditary and familial forms of cancer; the immuno-antitumor treatment of benign and malignant tumors; immunotherapeutic effect on the treatment of non-cancer diseases, such as limiting the development of bacterial or viral infections; and the possible triggering of the anti-aging process. In other words, the antitumor vaccine may be used as a promoter transferring a high level of functioning to the organism's immune system as a whole. Against this background, the probability of manifestation of the tumor process in individuals with preexisting risks of its development (genetically determined, enriched heredity, old age, etc.) would be minimized. Experimental implementation of these ideas, with a focus on effective clinical practice, has been demonstrated. For instance, in certain strains of mice with genetically determined breast cancer (C3H mice), a preventative (i.e., before the development of pathology) application of fetal liver cells leads to a decrease in the frequency of tumor development and an increase in animal survival [11]. Furthermore, cryopreserved fetal progenitor cells exhibit a more pronounced antiblastoma effect than native fetal progenitor cells. In the last 10 years, impressive experimental and translational successes in molecular diagnostics of the predisposition to the development of oncologic pathologies have been achieved, opening up the prospect of cryo-based preventative approaches to treating oncologic pathologies.

Finally, in one study, a stable frozen zone volume was demonstrated *in vitro*, and its length, depth, and cryogenic margin determined using a standard medical ruler and Vernier caliper after freezing at −180 °C, using liquid nitrogen to provide cooling and freezing of a small portion of this solution in the vessel at room temperature (20 °C). The application of fractal theory for assessing the distribution efficiency of contrast medium and therapeutic substance(s) in living biological structures for diagnostic purposes and for selecting a compassionate treatment strategy in medical professional practice has been proposed [88,89,140].

9. Conclusions

In summary, the new field of autologous and allogeneic cryoimmunological medicine is one of the hottest topics in a wide range of experimental and clinical studies aimed at the analysis of the pathogenesis and development of methodological approaches for the treatment of malignant neoplasms. Immunotherapy has profoundly impacted cancer treatment, yet one of its greatest achievements to date has been the manner in which it has propelled cancer research. Undoubtedly, this particular branch of scientific thought of clinical experience will enable a global leap towards increasing the effectiveness of antitumor immune therapy in various fields of medical practice in the near future.

Authorship

N. N. K. and A. N. G. were responsible for the conception and design of this review, and analyzed and interpreted the manuscript data. O. I. D. and M. O. B. contributed to several subsections relevant to the subject of the manuscript. All authors prepared the initial draft of the manuscript, contributed to manuscript revision, and approved the submitted final version.

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Data availability

All relevant data are included in the paper.

Ethics

Written informed consent was obtained from patient with malignant melanoma in accordance with the principles of the Declaration of Helsinki, 1964, and its later amendments. The patient signed a declaration of consent for the clinical study that uses cryogenic diagnostics after detailed explanation with a surgeon-physician about the purpose, manner, and course of events of the planned interventional cryodiagnostics and surgical treatment, and this is documented in the clinical history of the patient. The Resolution on Compliance is available with Ethical Principles, identification number: EC_No. 10/2001.

Declaration of competing interest

None.

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