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CANCER XENOGENEIC VACCINES BASED ON CHICKEN ANTIGENS. WHY NOT?

Cancer vaccines are still attracting scientists' attention. The more and more articles devoted to the elaboration of cancer vaccines are published each year. To increase vaccine efficacy, new approaches are studied, including the use of xenogeneic antigens. ***Aim:*** *to summarize the main results obtained in the process of constructing xenogeneic cancer vaccine based on chicken embryo antigens.* ***Conclusion:*** *It was shown that EDTA-extract of whole chicken embryos contains at least 2 homologous proteins (MMP-2 and VEGF); it has anticancer effects on Lewis lung carcinoma and Ehrlich carcinoma models and elicits anticancer immune reactions. Although a lot remains to be elucidated, chicken embryo extract may be used in xenogeneic cancer vaccines engineering.*

Although different immunotherapeutical modalities are elaborated now, cancer vaccines still attracting scientific attention. Low toxicity, comparatively high specificity and long lasting memory let cancer vaccines remain on a list of promising modalities for decades. Even more, with the development of immune checkpoint inhibitors, a new important role cancer vaccines can play emerged. In order to facilitate the effectiveness of immune checkpoint inhibitors, specific T-cell immunity should be activated first [1]. Cancer vaccines perfectly fit the role of specific immunity activators. So, despite limited clinical success, the field of cancer vaccines engineering is constantly growing. To illustrate, for the search words «cancer vaccine» PubMed service retrieved 430.560 results, of them in 2018, 2019 and 2020, respectively, 2.848, 2.878 and 3.533 articles were published.

Among cancer vaccines there are xenogeneic ones, which utilize xenogeneic homologous proteins as antigens to elicit anticancer immune response. They are believed to be able to break immune tolerance towards cancer antigens. It is supposed that highly homologous although not identical antigens can be sensed as an «altered self» by immune cells breaking thus immune tolerance towards self-antigens. As some authors propose [2], xenoantigen should share about 85–95% of homology to avoid undesirable T-cell response amplification, on the one hand, and to induce effective cross-reactive immune reaction, on the other. But actually xenoantigens shearing 81% [3], 71% [4], and even about 60% [5, 6] have been examined and were shown to be able to induce immune activation sufficient enough to impede tumor progression. So, it looks like not simply homology level plays the main role, but in which part of the protein molecule the difference exists. It is considered that efficient xenogeneic antigens contain so called heteroclitic epitopes with a higher affinity for MHC molecules [7, 8]. Therefore, it looks like a potent immunoactivating xenogeneic antigen can be chosen by means of *in silico* modeling. Actually, there are some works exploring this kind of approach, selectively replacing certain amino acid residues in the antigen molecule strengthening thus MHC class I binding. They are called heteroclitic peptides or MHC anchor-modified ligands [7–10]. Another approach is based on engineering of chimeric proteins that include both homologous and xenogeneic moieties [11]. Nevertheless, possibly because of the fact that «the methodical selection of MHC class I target epitopes and the design of heteroclitic peptides are complex and time-consuming tasks» [9], the majority of the research on xenogeneic cancer vaccines is based on proteins or genes of different species origin. For instance, from xenopus [12], rat [13, 14], mouse [3, 15], rhesus [16], bovine [17], porcine [18, 19], quail [20], chicken [4, 21–25], etc. In our laboratory at R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, we studied proteins of chicken origin as candidate antigens for xenogeneic cancer vaccine engineering.

A xenogeneic antigen to be used as a component of vaccine should meet some obvious requirements: it should be tightly associated with cancerous process (cancer associated antigen, angiogenic factor, enzyme involved in cancer progression etc) and share high homology with the protein the vaccine is targeted at. Many proteins taking part in the essential processes as embriogenesis and tumorogenesis are highly conserved among different species. Indeed, searching in the literature and in the BLAST (Basic Local Alignment Search Tool, accessible at https://blast.ncbi.nlm.nih.gov/Blast.cgi), we find more than 15 proteins of chicken origin which share high gomology with human counterparts (Table).

Moreover, chicken embryo expresses proteins shearing homology with human nuclear pore complex protein Nup88 which is overexpressed in a variety of human tumors [34] and MAGE-like protein [35].

Currently, various antigens of chicken origin were examined as xenogeneic cancer vaccines [4, 21–25, 36], demonstrating some promising results. To avoid immune editing [37], we started to test whole chicken embryo extract as an antigenic part of a future xenogeneic cancer vaccine.

Table

Some proteins of *Gallus gallus domesticus* sharing high gomology with the corresponding proteins of *Homo Sapience*

№	Protein	Homology level	Process involved in	Reference
1.	MMP-MT3	89.0%	Metastasizing, invasion	[26]
2.	MMP-2	84.0%	Metastasizing, invasion	[27]
3.	MMP-13	76.0%	Metastasizing, invasion	[26]
4.	MMP-9	59.0%	Metastasizing, invasion	[26]
5.	Flt-1	65.0–95.5% depending on the domain	Angiogenesis	[28]
6.	FGFR-1	92.0%	Angiogenesis	BLAST
7.	Angiopoietin-1	91.0%	Angiogenesis	[29]
8.	Angiopoietin-2	87.0%	Angiogenesis	[29]
9.	Tie-2	71.0%	Angiogenesis	[4]
10.	VEGF A	75.0%	Angiogenesis	BLAST
11.	sVEGFR1	74.0%	Angiogenesis	[30]
12.	Survivin	~60.0%	Inhibitor of apoptosis, highly expressed in most human tumors and fetal tissue	[31]
13.	CTK-1	95.0% comparing to the insuline receptor	Growth factor	[32]
14.	CTK-2	94.0% comparing to the IGF-1 receptor	Growth factor	[32]
15.	SPARC (osteonectin)	85.0%	Expressed by wide variety of cells, including numerous neoplastic cell	[33]

The application of embryonic/fetal material in cancer research has a long history in detail overviewed in [38]. Among others, it was shown that immunization with embryo extracts can suppress the growth of experimental tumors and protect from tumorigenesis caused by viral and chemical agents. Embryogenesis and tumorigenesis share some similarities in terms of expressed antigens, activated molecular cascades, involved enzymes etc [39, 40]. This resemblance is even more striking for cancer stem cells. For example, 33 out of 40 currently known surface markers of cancer stem cells are expressed on embryonic or adult stem cells, and are rarely detected on normal tissue cells [41]. So we hypothesized that using embryo extract of xenogeneic origin we could break immune tolerance towards antigens sheared by embryo and cancer cells targeting cancer stem cells too.

For the investigation, EDTA extract of a 7-day chicken embryo was used. Extraction method which was appied allows obtaining predominantly extracellular proteins [42], for example fibronectin [43] and transferrin [42]. At least 15 bands were seen after not denaturating electrophoresis of our resulted extract which we called chicken embryo proteins (CEP).

During embryogenesis, metalloproteases and angiogenic factors are intensively produced. Moreover, they are secreted extracellularly, so could be extracted by the method we used. To investigate whether the extract contains metaloproteases 2 and 9, zymography in 10% polyacrylamide gel with 0.1% of gelatin was applied. Two bends corresponding to 71.9 and 62.8 kDa were detected on the zymogramme [44]. The gelatinolytic activity was blocked by EDTA which belongs to specific matrix metalloproteinase (MMP) inhibitors [45]. Therefore, we concluded that the extract contains active (64–62 kDa) and zymogene (70–72 kDa) forms of MMP-2 [27, 46]. VEGF was confirmed to be present in CEP with ELISA [44]. So we obtained extract containing at list two proteins playing important role in cancer progression shearing high homology with the human counterparts (84% and 75% for MMP-2 and VEGF respectively). Both MMP-2 and VEGF of different origin were studied as means of the xenogeneic vaccination by different researches and were shown to elicit crossreacting immune response that resulted in anticancer effect [21, 24, 47, 48]. In some studies, even allogeneic VEGF-based vaccines were able to induce VEGF-specific antibodies which blocked angiogenesis inhibiting thus cancer progression [49–51]. Therefore, obtaining the protein mixture encompassing at least two promising antigens we were inspired for further investigations.

On the first step, immunogenisity and toxicity of the CEP in mice were checked [52]. Mice representing different types of immune response [53] were used: regarded as Th1-dominant C57Bl mice and apt to Th2 response Balb/c strain. CEP-specific IgG were detected in 91.7 ± 7.9% (11 out of 12) and 68.2 ± 9.9% (15 out of 22) of Balb/c and C57Bl mice respectively. As it was studied on blood analysis, body weight changes and the percentage of viable immune cells in thymus, spleen and four lymph nodes, administration of CEP did not produce toxic effect in mice of both strains. Therefore, we considered the chicken embryo extract as safe and feasible means for further investigation.

On the next step, anticancer effect of CEP was studied on 3 experimental tumors: Lewis lung carcinoma (LLC), Ehrlich carcinoma (EC) and sarcoma 37. Anticancer effect of the CEP applied in different settings (before the tumor challenge, after the tumor cells transplantation and after the removal of tumor nodule) was studied.

It was shown that CEP anticancer effect depended on both experimental tumor and immunization settings. There was no statistically significant anticancer effect on sarcoma 37 tumor independent on the immunization schedule. On the other hand, anticancer effect was seen on carcinomas — LLC and EC tumors [54–56].

First, we studied the anticancer effect of CEP applied before tumor cells transplantation. In order to exclude that anticancer effect of CEP may be produced by unspecific immune inflammation (resembling the approach used to treat cancer by *Coley* [57]), we deliberately used a schedule which implies the tumor challenge on day 30 after the last immunization. Till the day 30 after the last CEP injection, the immune response induced

by the immunization was expected to terminate, but the immune memory cells would have remained. It was shown that applied before tumor cells injection (3 times with 7 days interval), CEP significantly prolonged the latent period of tumor outgrowth (by 39.7%) and inhibited its growth. In the group of immunized LLC-bearing mice, latent period of tumor outgrowth increased by 39.7%, the Index of Tumor Growth Inhibition reached 35.8–48.8% depending on the day of tumor growth and was significant as compared to the unimmunized tumor-bearing control over the entire experiment (till day 28 of tumor growth). Possibly, as a consequence of tumor growth inhibition, metastases number and volume were decreased in the immunized mice too. Metastases Inhibition Index in the group reached 71.1% [55]. Interesting enough, used as a control, frozen-melting extract of LLC prolonged latent period of tumor outgrowth but had not any anticancer effect. To some extent, this finding goes in line with the results of other scientists reporting xenogeneic vaccines often have superior efficacy as compared with autologous or allogeneic counterparts [4, 6, 13, 16, 36, 58].

In the group of mice immunized before the EC challenge, latent period of tumor outgrowth was prolonged by 17.9%, Index of Tumor Growth Inhibition was 17.4–48.1% (in both cases, $0.05<p<0.1$ as compared to the unimmunized control) [44].

For immunizations after the tumor challenge, different schemes of CEP administration were tested in order to choose the most efficient one. It turned out that notable anticancer effect depended on the experimental tumor and appeared when immunization had started shortly after the tumor challenge. In the case of EC tumor, 50.0% of tumor growth inhibition ($p < 0.05$, days 13–16 of tumor growth) was reached when immunization had been performed on days 2, 5 and 8 after the tumor transplantation [56]. In LLC, tumor growth inhibition by 53.0% ($p < 0.05$, day 14) and metastases inhibition index by 77% was reached when the immunization had been done on days 1, 7 and 14 after the tumor challenge [55]. In other words, immunization with the CEP proved to be effective when started on the settings of a low tumor burden. This is understandable as long as growing tumor imposes immune suppression resulting in an inability of immune system to eliminate cancer cells efficiently. The fail of cancer vaccines trials can be attributed to enrolment of patients with the huge tumor burden [59]. So, it can be concluded that cancer vaccines can hardly be effective when applied as a monotherapy or without prior tumor debulking. Therefore, to reach the maximum effect, an anticancer vaccine should be applied in less aggressive disease settings, that are in minimal residual disease.

Indeed, a potent and long-lasting antimetastatic effect was reached, when CEP was applied after the surgical removal of LLC tumor. The Metastases Inhibition Index, compared to the control mice which underwent only surgery, reached 96.9% and 97.8% on days 18 and 34 after the tumor removal respectively [55]. These results perfectly support a generally accepted assumption that the main goal of cancer vaccines application is prevention of metastasis and relapses after primary tumor resection [59, 60].

To unravel mechanisms underlying the anticancer effect of CEP, immunological analyses of the immunized and control tumor-bearing mice have been carried out. For that, LLC- and EC-bearing mice were immunized according to the most efficient immunization scheme, and immune reactions were checked on days 7, 14, 21 and 28 of tumor growth. Immune reactions in mice immunized after surgical removal of LLC tumor were studied too.

It was discovered that the immune effects of CEP application to LLC- or EC-bearing mice were different. In the mice immunized after the LLC challenge, CEP application elicited both natural killer (NK) cells and cytotoxic T-lymphocytes (CTL) activation. Compared to the untreated tumor-bearing mice, NK cytotoxic activity (CTA) of immunized mice was by 61.5% higher on day 7 after the tumor challenge ($p < 0.05$); CTL CTA was by 94.9%, 49.4% and 164.3% higher on days 14, 21 and 28 respectively ($p < 0.05$); lymphocyte proliferation induced with the antigens of LLC cells was by 94.4%, 49.7%, and 163.4% higher on days 14, 21 and 28 respectively; antibody-dependent lymphocytes CTA was 388.9%, 114.0% and 246.5% higher on days 14, 21 and 28 respectively ($p < 0.05$) [44]. In the immunized group, the elevation of NK CTA on day 7 coincided with a sharp IFN-γ increase in blood serum, which exceeded the intact mice IFN level by 9.4 times ($p=0.08$); IFN/IL-4 ratio in the group of immunized mice reached 81.7, whereas in the control tumor-bearing group it made 36.9, and in the intact group it was only 10.2. It is known that NK cells provide an early source of IFN-γ which is crucially important for the polarization towards Th1 immune response [61]. In our experiment, activation of NK cells and increase in «early» IFN precede the activation of Th1 type immune response in the immunized mice. It remains to be elucidated how NK cells were activated with CEP as long as these cells, to our knowledge, cannot be activated with soluble antigens. On the other hand, NK cells can be activated through the cross-linking of their Fc-receptors (mainly CD16) with antibody-antigen complexes. The presence of CEP-reacting antibodies in the blood serum of tumor-bearing mice was shown in our previous experiments [54]. Therefore, we assume that interaction of CEP-antibodies immune complexes with NK Fc-receptors could lead to the activations of these cells. Even more, in some circumstances, as parallel stimulation of NKG2D and CD16 receptors or combined IL-2/IL-18 stimulation, NK cells can gain APC-like properties [62, 63], and therefore can serve as a bridge between innate and adaptive immunity. LLC-cells do naturally express ligands for NKG2D receptor [64] and therefore could further stimulate previously activated NK-cells turning them into APC-like cells. But, this premise warrants further investigation. If it is re-

ally so that pre-existing CEP-specific antibodies play some role in NK-cells activation, the presence of pre-existing CEP-specific antibodies possibly may be used as a screening biomarker to predict «responsiveness» to CEP-based vaccine.

Immunization with CEP after the surgical LLC removal protected NK cells from surgery-induced down-regulation in the early postoperative period [65]. Surgery is known to impose stress on immune cells and on NK cells particularly [66, 67]. Indeed, on days 7 and 14 after the tumor resection NK CTA of the control mice, which underwent surgery but got no immunization, was by 2.0 and 2.5 times lower compared to the intact control ($0.05 < p < 0.1$ and $p < 0.05$, respectively). On the contrary, in the group of immunized mice, suppression of NK CTA was postponed and evident only on day 14 after the tumor removal. There is evidence that the perioperative application of immunoactivating remedies can protect from metastatic spread of the cancer cells caused by the surgery [67–70]. Indeed, during the follow-up period (day 21–38 after the tumor resection) there were no metastases detected in the immunized group of mice, contrary to the control group where 6 out of 10 mice developed metastases.

Moreover, in the group of immunized mice the increase in spontaneous lymphocytes blast-transformation (which is an unspecific index of *in vivo* lymphocyte activation) was seen. It was significantly higher than that in the intact control group (by 56.8%, 24.3% and 43.9% on days 7, 14 and 21 correspondently) [65]. This effect, at least partially, could enable faster recovery from the surgery-imposed stress in the immunized mice. Taking together, we concluded that immunization with CEP after the resection of primary tumor protects the immune system from the surgery-imposed stress and accelerate the recovery preventing thus metastatic cancer spread. It is tempting to say that the proper timing of vaccination and prior tumor debulking are contributing to the anticancer effects of the vaccination more than the magnitude of immune response elicited by the vaccination.

Applied to EC-bearing mice, CEP induced antibodies production [56]. The number of antibodies-producing immunized mice was increasing over the entire experiment; on days 21 and 28 of tumor growth all the immunized mice produced antibodies against antigens of EC and CEP ($p < 0.05$ as compared to the control tumor-bearing mice). The level of CEP-specific antibodies was continuously growing and on days 21 and 28 it was higher ($p < 0.05$ and $p < 0.07$ respectively) than that in the control tumor-bearing group. The level of antibodies specific to antigens of EC did not differ significantly between the groups. The produced antibodies probably were involved in the reactions of macrophages' antibodies-dependent cytotoxicity which was slightly elevated in the immunized mice as compared to the tumor-bearing control.

In EC-bearing mice, immunization with CEP brought about another interesting effect: it possibly protected macrophages from type 2 polarization [71]. In the control tumor-bearing group, macrophages lost cytotoxic activity and produced less IL-1 but more IL-10 as compared to the intact control. Moreover, the downregulation of macrophages activity inversely correlated with the level of the medium size circulating immune complexes ($r = -0.71$, $p = 0.03$) and IL-4 ($r = -0.59$, $p=0.07$) (both are known to polarize macrophages towards 2 type). On the contrary, in the immunized group, macrophages' direct and antibodies-dependent cytotoxic activity were elevated ($p < 0.05$) as compared to the intact (on day 7 of tumor growth) and the tumor-bearing control (on days 14 and 28) groups. Moreover, macrophages of the immunized mice actively produced TNF-α, reaching the peak on day 21. Altogether these findings point to the type 2 macrophages polarization in the control tumor-bearing group and to the type 1 polarized macrophages in the immunized group. Of course, this result remains to be elucidated with the other methods, but in case it is proved, keeping macrophages classically activated is a very promising effect of immunization with CEP. As long as type 2 macrophages favors tumor progression, to polarize macrophages towards anticancerogenic type 1 is of crucial importance for effective cancer treatment and currently several approaches modulating macrophages activity are tested as cancer treatment modalities [72].

So, the immunization with CEP has an anticancer effect in mice bearing LLC or EC tumors. The immunological reactions underlying the anticancer effects differed and probably depended on the mice' genetic background as well as on the cancer model. Generally speaking, in C57Bl mice which are tending to Th1 immune response, CTL and NK cells activities were elevated after the immunization, on the contrary, Th2 biased Balb/c mice increased antibodies production. The multiplicity of immune reactions elicited by the CEP immunization, to our mind, was possible due to the poliantigenic nature of the embryo extract.

So, where does go the further road of the xenogeneic chicken-embryo based vaccine elaboration? Effectiveness of any vaccine and cancer vaccine in particular depends on the proper choice of the adjuvant [73]. As an initial attempt, we tried combining CEP with TLR2/4-stimulating Bacille-Calmette-Guérin (BCG), which itself demonstrates anticancer activity in bladder cancer patients [74] and in some cases was used as an adjuvant in cancer vaccines formation [75–77]. Unfortunately, we did not reach additional or synergetic effect [78]. This result may imply at least two consequences: 1) an inappropriate adjuvant was chosen, the search for an optimal one should continue or 2) xenogeneic antigens are potent immunoactivators by themselves, so they need no adjuvants at all. There is still another way of CEP vaccine improvement. A combined or prime-boost scheme of xenogeneic and autologous vaccines application is poorly elucidated as for the cancer vaccines in general and for the CEP-based vaccine in particular. So further researches are urgently needed and worth doing.

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ПРОТИПУХЛИННІ КСЕНОГЕННІ ВАКЦИНИ НА ОСНОВІ АНТИГЕНІВ КУРКИ. ЧОМУ НІ?

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Резюме. *Протипухлинні вакцини надалі привертають увагу вчених. З кожним роком публікується все більше статей, присвячених розробці вакцин проти раку. Вивчаються нові підходи для підвищення їх ефективності, включно з використанням ксеногенних аналогів антигенів.* ***Мета:*** *підсумувати основні результати, отримані в процесі створення ксеногенної протипухлинної вакцини на основі антигенів ембріона курки.* ***Висновки****: показано, що ЕДТА-екстракт ембріона курки містить щонайменше 2 гомологічні білки (MMP-2 та VEGF), виявляє протиракову активність на моделі карциномі легені Льюїс і раку Ерліха та активує імунні реакції, які беруть участь у протипухлинному захисті. Незважаючи на те, що досить багато аспектів ще потребують детального вивчення, екстракт ембріона курки може бути використаний для розробки ксеногенних протипухлинних вакцин.*

Ключові слова: ксеногенна протипухлинна вакцина, екстракт ембріона курки, протипухлинна ефективність, протипухлинні імунологічні реакції, карцинома легені Льюїс, карцинома Ерліха.

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