Review

The application of circulating tumor cells detecting methods in veterinary oncology

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Abstract

Cancers are one of the most common diseases affecting dogs. Many of them develop sponta-neously and their biology and histopathology shows many similarities to human cancers. What more, it is proved that there are much more analogies in molecular mechanisms of cancer development between these two species. Human oncology is seeking more and more efficient methods for an early disease detection which results directly in the extended life expectancy of patients affected. One of the most modern trends in the diagnosis of cancer is to detect circulating tumor cells (CTC) in the blood of patients. It is known that these cells are responsible for the formation of metastases in distant organs what results in the patient death. Moreover, it's confirmed that CTC are already present in patients' bloodstream in the early stages of tumor development. There is no doubt that mechanism of metastasis development in dogs is identical and thus the CTC are also present in their bloodstream. Despite the intense researches there is still no optimal method of isolating cancer cells from the blood where they occur extremely rarely. The purpose of this study is to analyze the implications of the detection methods of tumor cells in the blood in veterinary oncology.

Key words: circulating tumor cells, cancer, metastasis, dog

Introduction

Tumors are the major cause of the morbidity and mortality in dogs. In contrast to other domestic ani-mals (except cats) dogs, as pet animals, live long enough to develop spontaneous tumors, analogically to humans. In addition, some breeds are genetically susceptible to certain types of tumors, as in case of boxers and rottweilers. Of course, dogs do not smoke and do not use excessive amounts of salt so they are not exposed to the major carcinogenic factors experienced by humans. However, dogs, living close to humans, are exposed to the same environmental fac-tors which are linked to increasing occurrence of tu-mors in the man.

The occurrence of tumors in a dog is a significant

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problem in veterinary medicine. The occurrence of neoplastic disease in animals being often regarded as the members of the family imposes a big demand on the development of effective methods of the diagnostics and therapy. Although the surgery is a method of choice in the most cases, the owners often do not refrain from choosing more expensive methods of treatment, as chemotherapy or radiotherapy. This propels the significant progress in veterinary oncology.

On the other hand, dogs can be a good model of human tumours. Saying nothing about beagles, being known to be particularly resistant to carcinogenesis, many types of tumors in dogs are developing in a way similar to that in humans and are histopathologically very similar (MacEwen 1990). A good example is mammary gland cancer (Gilbertson et al. 1983), transitional cell carcinoma (TCC) of the bladder (Knapp et al. 2000) and squamous cell carcinomas (Henry et al. 2005).

Moreover, there is an evidence that there are far more similarities at the molecular level of the disease development (Rowell et al. 2011). The genetics of the domestic dog is more close to the human genetics than these of other animal species (except primates). The dogs suffer more from naturally occurring hereditary diseases than any other domestic species (Patterson 2000). Among 450 hereditary diseases of the dog over 360 is analogous to human ones (Parker et al. 2004). Therefore, dogs are natural model for human hereditary diseases and provide an excellent system for the study of loci responsible for the emergence of disease, among them the genes responsible for carcinogenesis (Shearin and Ostrander 2010).

Naturally occurring cancers in dogs and humans share biological features, including molecular targets, telomerase biology and tumor genetics. It is becoming clear that the molecular drivers for dog and human cancer are analogous with near identical specific genes changes in the oncogens and tumor suppressor genes. As an example, a recently constructed syntenic karyotype map between dog and human demonstrated strong similarities in the cytogenetic abnormalities in Non-Hodgkin Lymphoma (NHL) occuring in both species (Pang and Argyle 2009). NHL is one of the examples of the environmental impact on the formation of cancer as well (Hayes et al. 1995). Dogs share the same habitat as man, making possible that the etiology of diseases in dogs is similar to humans. Despite sharing a common living space dogs do not drink alcohol, do not smoke (unless passively) and usually are fed properly. However, there is an evidence of the impact of such environmental factors as passive smoking, pesticides or asbestos (Glickman et al. 1983, Glickman et al. 1989, Reif et al. 1998) on the development of cancer in dogs.

Epidemiological studies of cancer incidence among dogs, carried out in the 90s of the twentieth century in Norway, have shown a direct effect of age and sex on the dominant type of cancer in the canine population. During two first years of life cutaneous histiocytoma was the dominant tumor type in both sexes. At the age of 2 to 4 years, in males histiocytoma was still the most frequent, while in females it was surpassed by benign epithelial skin tumors. After the age over 4, benign epithelial skin tumors were dominant in males, and mammary tumors in females. For animals from 4 to 14 years old tumour frequencies were slightly different. The most common were skin cancers (mastocytomas), followed by lymphomas. With the age the number of cancers related to sex increased. More testicle cancer cases were reported in males and mammary gland cancer cases were reported in females. In conclusion, the population study shows that various skin cancers were the most frequent disease and comprised 51% of all monitored cases, regardless of the animal gender. An interesting fact is that the mammary gland neoplasms in females were 30% of all neoplasms cases in the population. Males suffered also from testicles and skin cancer. Comparative research conducted in the same study showed that the mammary and testicular neoplasia seemed to be more frequent in dogs than in humans, while intestinal, pulmonary and prostatic malignancies were less common in dogs, each of them was under 1% of total (Gamlem et al. 2008).

The diagnostics and treatment of tumors in the dog is undoubtedly as difficult problem as in human. It is already known that in their development these tumors mimic human neoplasms. Human oncology offers variety of modern methods of detection and treatment of neoplasms, including cancers. For several years the extensive research carried out on the mechanisms of metastases formation concentrated on circulating tumor cells(CTC). The modern understanding of cancer biology and process of metastasis suggest that these circulating cells play a vital role in forming metastatic disease (Metge et al. 2008).

The ability to identify, isolate and characterize CTC subpopulation at the molecular level could further the discovery of cancer stem cell biomarkers and expand the understanding of the biology of metastasis (Nagrath et al. 2007).

Circulating tumor cells (CTC) – basic knowledge

The prognosis in carcinoma patients, even with small primary tumors, is still hampered by metastatic relapse frequently occurring years after diagnosis and

complete resection of primary tumors (Pantel et al. 2008). Since the rise and growth of metastases in distant organs during treatment of cancer in human is the cause of about 90% of deaths, identification and control of metastases is one of the most important trends in oncology (Fidler 1999). It is now known that the spread of metastases can occur even at an early stage of primary tumor (Husemann et al. 2008). Small neoplastic changes which escape the available diagnostic methods spread as circulating tumor cells (CTC) through the lymphatic or blood vessels. Already over 100 years ago an Australian physician-pathologist TR Ashworth has detected tumor cells in the peripheral blood (Ashworth 1869).The presence of CTC in the blood justifies the "Seed and soil" hypothesis of the metastases formation (Fidler 2003). It says that carcinoma cells escape from the primary tumor into the bloodstream and thereby migrate to distant organs where they can implant and create metastases. According to this theory, the fate of CTC may differ depending on their molecular profile. It has been calculated that one gram of tumor tissue is releasing about $1x10^6$ tumor cells every day (Chang et al. 2000). However, these cells undergo the elimination processes (Glinsky et al. 2003). About 85% of these cells disappear from the bloodstream in less than 5 minutes (Berezovskaya et al. 2005). So rapid disappearance of carcinoma cells is associated with a process of programmed death, called Anoikis process, due to the lack of cell contact with intercellular matrix. Not without significance is also the process of CTC elimination from the blood by cytotoxic lymphocytes. Cells in which malignant transformation went far enough to wean themselves from contact with the matrix, and which are not recognized and attacked by the immune system, are capable to pass through the vessel wall and create micrometastases. *In vivo* studies in animals have shown that only one out of 40 cells that survived the elimination are able to form micrometastases and about 0.01% of them develop into tumors at the macro scale (Luzzi et al. 1998). To identify these rare cells in the blood sensitive and specific assays have been developed (Pantel et al. 2009).

Circulating tumor cell characterization methods

Most existing research focused on the development of an efficient and reproducible methods for detecting CTC. Specific markers for these cells, coming from different malignant neoplastic lesions, are sought. As it is well known carcinomas are tumors of epithelial origin and,therefore, most common strategy

in circulating carcinoma cells detection is to use markers specific for these cells. The most obvious feature is their size and shape, diametrally different from the blood cells. However, cytokeratins (CK), cytoskeletal epithelial cells proteins, characteristic for epithelial cells and EpCAM adhesion protein of epithelial cells are used most often as specific markers. Moreover, markers specific to various types of cancer such as prostate specific antigen (PSA) (prostate cancer) and mammoglobin (breast cancer), are used.

The ideal method should be highly sensitive, reproducible and easy to use. The CTC concentration in the blood is low, defined as 1 cell per 10^6-10^7 leukocytes (Mostert et al. 2009) and for this reason enrichment stage is often required to raise the sensitivity of assay. This step is followed by the detection stage.

The multiannual research of CTC detection methods has led to the following strategies: enrichment method followed by detection methods, and combined methods which are mixing both enrichment and detecting methods.

Circulating tumor cells enrichment methods

All the techniques of CTC enrichment from blood rely on the use of a natural properties of these epithelial cells.

Filtration

The simplest but regarded the least specific (Attard et al. 2011) is filtration (called "the isolation by size"), of epithelial tumor cell (ISET). It involves the use of polycarbonate filter which pore diameter is smaller than 8 μm (Tao et al. 2011). The method assumes that the nucleated blood cells and red blood cells are separated from the CTC because their diameter is less than the diameter of the pores of the filter. Unfortunately, this cheap and simple method is the least sensitive. This is due to the fact that some leukocytes may reach a diameter greater than 8 μm and often contaminate the sample.

Density gradient

Another method that uses natural properties of the cells is the density gradient centrifugation. It is assumed that with suitably chosen gradient (Ficoll-Paque™) heavier cells, as leukocytes and erythrocytes, form pellet at the bottom of the tube

during the centrifugation. Because of their weight and shape CTC can be found in the supernatant (Kruck et al. 2012). An upgraded version of the simple gradient is the OncoQuick® (Greiner Bio One, Germany). This 50 ml centrifuge tube contains the properly selected gradient, but also include semi-permeable barrier that separates unwanted cells from the CTC collected at the interphase (Obermayr et al. 2010).

Immunomagnetic particle

This enrichment method is one of the most commonly used. It uses a magnetic immunobead and ferrofluid. They are conjugated with antibodies to such antigens as: cytokeratin (CK), epithelial cell adhesion molecule (Ep-CAM) or human epithelial antigen (HEA). In general, they are based on the positive and negative selection. Positive selection uses beads bound to antibodies to antigens such as: cytokeratins (CK), epithelial cell adhesion molecule (EpCAM), or cancer markers: carcinoembryonic antigen (CEA) and epidermal growth receptor 2 (HER2).The immunomagnetic particles connected with cells are separated from the rest of the cells in a magnetic field. In the case of negative selection particles are coated with antibodies to CD45 and CD61, which are leukocyte and megakaryocytes surface markers. In this way the entire population of unwanted cells is removed from the solution. The described method of isolation seems to be very promising, however, it suffers from several disadvantages. Among others, in the process of carcinogenesis epithelial cells can undergo the so-called mesenchymal conversion process (epithelial to mesenchymal transition-EMT), which leads to the loss of specific epithelial cells surface markers (Goeminne et al. 2000).

So far, several products based on this method have been developed:

Dynabeads® Epithelial Enrich (Invitrogen) (Reinholz et al. 2005) are superparamagnetic polystyrene beads (4.5 μm diameter) coated with a mouse IgG1 monoclonal antibody (clone Ber-EP4) specific for two (34 and 39 kDa) glycopolypeptide membrane antigens expressed on most neoplastic and normal human epithelial cells (Latza et al. 1990).

MACS®Magnetic Activated cell Sorting System (Milteny Biotec GmbH, Germany) are 50-nm superparamagnetic particles that are conjugated to highly specific antibodies to a particular antigen on the cell surface (Anti-Cytokeratin, CD326 (EpCAM), CD45). It makes use of microfluidics technology to achieve simultaneous spatially-addressable sorting of multiple target cell types in a continuous-flow manner (Kim et al. 2008). Enrichment with magnetically labeled anti-cytokeratin antibodies increases the detection rate of epithelial cells in the bone marrow of cancer patients compared to immunocytology (Weihrauch et al. 2002).

RosetteSep® (Stem Cell Technology,Vancouver, Canada). Negative selection involves the antibodies tetramers against markers, as CD2, CD16, CD19, CD36, CD38, CD45, CD66b and glycophorin A, which bind the antigens on unwanted cells. Thus, created complexes are separated from tumor cells during density gradient centrifugation in Ficoll-Paque™ (Beeton and Chandy 2007, He et al. 2008)

Circulating tumor cells detection methods

The basic markers are surface antigens detected by immunocytochemical methods and/or the presence and expression of individual genes.

Application of immunocytochemistry

It is the one most widely used among the detection methods. This is due to the fact that labeled cells can be not only counted (using cytometry) but also, if necessary, be observed using a microscope. It has therefore a decisive advantage in comparison with the gene expression study where the cells must be lysed. The main task in the cytometry methods is to select the optimal marker for CTC which must not be present simultaneously on blood cells, or normal epithelial cells appearing in the blood after surgery or in the inflammation (Crisan et al. 2000). Most commonly it is proposed to identify the cells on the basis of epithelial markers: EpCAM, CK, or BerEP4. It is known, however, that the carcinoma cells are capable of losing these surface markers which can lead to false negative results (Rupp et al. 2011). There are also attempts to use the fact that some cancers have a self-specific markers, such as mammoglobin (breast cancer), or prostate-specific antigen (PSA) (prostate cancer), but their expression is not consistent. The situation is further complicated, for example, in the gastric cancer which does not have a unique marker, but a few ones may be present with varying intensity depending on the patient (Fuse 2011, Janjigian and Shah 2011, Ochiai 2011). The ICC-based techniques are described below.

Laser Scanning Cytometer (LSC CompuCyte) uses a laser-based opto-electronics and automated analysis capabilities to rapidly measure biochemical constituents and simultaneously evaluate cell morphology. Although LSC technology is of a flow cytometry heritage, it is not limited to analyzing cells in fluids.

Instead, the technology allows automated analysis of solid-phase samples, including tissue sections, adherent cultured cells, cancer tissue imprints, and cytology smears, preserving the sample picture along with the exact position of each measured sample. This important feature allows the researcher to automatically return to and visually inspect specific cells having defined biochemical or morphological properties (Zabaglo et al. 2003).

FAST® (Fiber-optic Array Scaning Technology), which applies the laser-printing techniques to the rare-cell detection problem. With FAST cytometry a laser-printing optics is used to excite 300,000 cells per second and emission is collected in an extremely wide field of view (Krivacic et al. 2004).

Multiphoton intravital flow cytometry is a method that noninvasively counts rare CTCs *in vivo* as they flow through the peripheral vasculature. The method involves intravenous injection of a tumor-specific fluorescent ligand followed by multiphoton fluorescence imaging of superficial blood vessels to quantitate the flowing CTCs (He et al. 2007).

Application of nucleic acid-based detection

Detection methods based on nucleic acids characterization potentially provide the widest application. The only limitation is the knowledge of analyzed nucleotide sequences. In this case the method is based on genomic DNA or gene expression investigated at mRNA level. The DNA is much less frequently used as a marker for CTC. Firstly, there is no specific mutation in the DNA for each CTC's. Secondly, there is an evidence of the presence of a cell-free DNA in the blood released from dying cells which, in comparison to the RNA molecule, is more stable (Wu et al. 2002). This property of DNA leads to a limitation in its use as it could lead to false positive results. However, few groups are looking for a relationship between the level of free DNA in the blood in healthy individuals and those suffering from cancer (Giacona et al. 1998, Schwarzenbach et al. 2009). In spite of all problems CTC can become "a biopsy material" on which one can perform FISH or PCR-RFLP analysis of genomic DNA to detect mutations charcteristic for a specific disease scenario and adapt appropriate methods of treatment. One example may be epidermal growth factor receptor (EGFR) mutations in non-small cell lung cancer (Maheswaran et al. 2008, Leary et al. 2011).

The vast majority of experiments are associated with messenger RNA. The occurrence of specific RNA in a cell is closely related to its activity profile. The mRNA allows for distinguishing epithelial cells from blood cells, such as lymphocytes, by selecting the appropriate markers. Epithelial cells are displaying the expression of several previously mentioned proteins (eg CK8, CK18, CK19, EpCAM) which other cellular blood components do not show. The basic method is a reverse transcription PCR (RT-PCR) (Kruck et al. 2011, Saloustros et al. 2011, Wu et al. 2011). With this method it is possible to demonstrate the expression of sought genes. Modification of this method, more often used to detect CTC, is Real-Time PCR allowing to define not only the presence but also to determine the gene expression levels (Andrusiewicz et al. 2011, Devriese et al. 2011, Molloy et al. 2011, Pilati et al. 2011). Of course, detection methods based on nucleic acids also have some drawbacks. Firstly, the methodology of DNA or RNA preparation for analysis requires cell lysis which prevents further investigations performed on whole cells. Moreover, high sensitivity may, paradoxically, lead to false positive results (Ballestrero et al. 2005).

Combined methods of enrichment and detection of the circulating tumor cells

This group includes methods connecting the two stages of isolation and characterization of circulating tumor cells.

CTC Chip technology® is unique because of its use of microfluidics provides a platform by which one can vastly increase the sensitivity and yield of capturing rare cell populations from whole blood, while doing so in a gentle manner that preserves the viability of isolated CTCs. The CTC-chip is a silicon chip of the size of a standard microscope slide on which an array of 78,000 microposts is etched with a specific geometric pattern and then the microposts are coated with antibodies against epithelial cell adhesion molecule (EpCAM). Whole blood is pneumatically pushed over the surface of the CTC chip and through the forest of microposts. The fluid dynamics imposed by the geometric arrangement of the microposts leads the cellular component of the blood down specific streamlines that are intersected frequently by the posts, thereby maximizing interaction of the CTCs with the anty-EpCAM antibodies. It results in a high-efficiency capture of the CTCs directly onto the sides of the posts. The captured cells can then be confirmed as CTCs (through staining which differentiates nonspecifically bound leukocytes from epithelial CTCs), counted, and further analyzed in a variety of ways including molecular characterization (Sequist et al. 2009).

Because the CTC-chip employs whole blood without any preprocessing and the sheer stress experi-

enced by CTCs as they travel through the chip is minimal, 98% of captured cells remain viable (Nagrath et al. 2007).

Microfluidic device. This system is a promising approach for efficient CTC sorting, analysis and culture in a completely integrated manner. New microfluidic system and strategy is allowing the sorting of cells, their study by confocal fluorescent microscopy and their subsequent culture (growth). The core of the system is a self-organized array of superparamagnetic particles coated with specific antibodies against surface antigens located in a microchannel. This original cell-separation microchip was integrated into a fully automated platform combining microfluidic technologies, microvalves and nanofluidic pneumatic pumps. Captured cells were fully viable and were *in situ* cultured (grown) in the array (Saliba et al. 2010).

The CellSearch System® (Veridex Co., Ltd.,Raritan, NJ, USA) is a semiautomated system for the detection and enumeration of CTCs that uses anti-CK antibodies, anti-EpCAM antibodies (both give a positive selection) and anti-CD45 antibodies (a negative selection). The CellSearch System® is based on a combination of immunomagnetic labeling and automated digital microscopy and is composed of several elements: CellSave Preservative Tube, CellSearch® Circulating Tumor Cell (CTC) Kit, CellSearch® CTC Control Kit, CellTracks® AutoPrep® System, CellTracks Analyzer II®. Cancer cells are labeled with antibody-covered magnetic beads that target EpCAM which is expressed on the cell membrane of tumor cells and immunomagnetically captured and concentrated. What more, cell nuclei are stained with 4',6-diamino-2-phenylindole (DAPI) and cells surface is labeled with anti-cytokeratin(CK) phycoerythrin (PE)-labeled antibodies (CK-PE). Negative selection includes anti-CD45 allophycocyanin-labeled (CD45-APC) antibodies, where CD45 is a leukocyte-specific antigen. Concentrated, stained tumor cells are examined by fluorescence microscopy to assess labeling by PE, 4',6-diamidino-2-phenylindole (DAPI) and allophycocyanin (APC) and thereby distinguishing tumor cells from leukocytes is possible. The CellSearch System® can detect 1 CTC per 7.5 ml of peripheral blood with a high reproducibility. This system was approved by the United States Food and Drug Administration in the monitoring of metastatic prostate cancer (MPC) patients. Currently it is cleared for monitoring metastatic breast and metastatic colorectal cancer patients (Park et al. 2011).

The AdnaTest BreastCancerSelect® *(AdnaGen, Langenhagen, Germany)* is another CTC test that features a CTC-enrichment procedure using a mixture of immunomagnetic beads coated with 1 of 3 antibodies to epithelial surface antigens. After enrichment, messenger RNA (mRNA) is extracted from obtained cells. The "number" of CTCs is indirectly determined by a semiquantitative RT-PCR method using probes for mRNAs specific for epithelial cell: MUC1, HER2, and the surface glycoprotein GA 733-2 and β-actin as a housekeeping gene (Ross and Slodkowska 2009). The test has the FDA approval for metastatic breast cancer monitoring (Fehm et al. 2010).

False positive results

Methods for determining the presence of CTCs in the blood are based on the more or less advanced techniques to capture them from the peripheral blood samples. Some methods (eg CellSearch) give the opportunity to observe morphology of the cells allowing for positive or negative assigning as a CTC. However, a number of methods based on markers for epithelial cells gene expression performed without visual identification, raise questions about the occurence of false positives results. The epithelial cells are absent in the blood under physiological conditions. The only cases they appear in the bloodstream besides the ongoing process of carcinogenesis are inflammation, trauma or previous operations. Therefore, in cancer patients the diagnostic tests in the direction of CTC are always shifted for a few weeks. Moreover, the most critical point in the correct determination of the CTC is the time of blood donation. Puncturing the skin and blood vessel endothelium may lead to inadvertent aspiration of epithelial cells. The simplest and most reliable way to avoid accidentally aspiration of epithelial cells is to reject the first 3-5 ml of blood. Adherence to the sampling regime allows for almost complete elimination of the possibility of false positive results (Saloustros et al. 2011).

Potential application in Veterinary Oncology

The basic problem with the use of the methods of detecting CTC in dogs is that they were designed and validated for use in humans. Although dogs are more related to humans than, for example rats, there are very significant differences at the molecular level, especially at the level of antigenic proteins. Although the molecules involved in the tumorigenesis are similar in humans and dogs and cancer cells express similar types of antigens as in humans the small differences in the aminoacid sequences may alter the structure of epitopes to the extent making them non-detectable with the antibodies used for their enrichment/detection in humans. The risk is higher because of the fact that the antibodies used in these methods

are most often of a monoclonal type and, as such, very highly specific and potentially non-reactive with canine antigens. It imposes a significant risk of the lack of the efficiency of the methods directly adapted from the human oncology. It seems that the biggest chance of success is associated with the methods based on the features for which the similarity between the two species is the greatest.

In general, the methods used in the canine oncology can be based on the same criteria as in human oncology: 1) based on cell morphology, 2) based on surface antigens, 3) based on nucleic acids.

Methods based on cell morphology

As previously mentioned, isolation, or enrichments, of epithelial tumor cells (ISET) may be achieved the "sieving" through the polycarbonate filter with a pore diameter which is smaller than 8 μm. This allows to eliminate erythrocytes because the canine red blood cells are approximately 7 μm in diameter, the same size as human cells (http://loudoun.nvcc.edu/vetonline/vet131/erythrocytes.htm). Obtained sample will be, however, contaminated by other, nucleated, blood cells (leucocytes). The other method of the enrichment, based on the gradient centrifugation, must be experimentally validated to give reliable results. The centrifugation medium is usually selected on the basis of literature data regarding the use in humans. There is a widespread use of cancer cells derived from immortalized cell lines to optimize the conditions of recovery of these cells, developed for testing the effectiveness of density gradient isolation.

The example of the commercial product suitable the enrichment of CTCs is OncoQuick® (Greiner Bio One,Germany), which in test cell recovery gives 69% – 91% reproduciblility and 1.08 – 1.83 cell per 20 ml of blood sensitivity detection limit (Gertler et al. 2003).

Methods based on surface antigens

Surface markers-based methods have always posed problems. Ideally, an antibody target should meet several criteria: it should be highly expressed by the cancer cells, it must be presented properly and stably on the tumor cell surface for its recognition by the antibody; it should be expressed by all, or nearly all, of the tumor cells and in a broad spectrum of different types of tumor; and it should be functionally involved in the tumorigenic process (Weiner and Borghaei 2006). As it is known, there is no universal marker that could be used to detect CTC of all types of cancers. Described previously different methods do not achieve 100% efficiency in CTC detection. This arises from the lack of the universal marker but also from epithelial-to-mesenchymal transition that alters the cell surface antigens profile (Giangreco et al. 2011). The evidence of that process in dogs may be the loss of cytokeratin 19 (CK19) expression in canine mammary malignant tumors (Gama et al. 2010). In the case of attempts to apply the CTC isolation methods in veterinary oncology problem may be the antibodies specificity to the antigens of the dog. "Cross-reactivity" is the ability of an antibody to recognize an antigen in a species different from the one where the antigen was isolated. It is extremely difficult to establish rules concerning Ab cross-reactivity. If the antigen is a protein or a glycoprotein, cross-reactivity usually decreases with phylogenetic distance – the higher the divergence between species, the higher the dissimilarity between orthologous genes. Linear or conformational epitopes on target antigens are easily lost as soon as one amino acid residue is different (Loisel et al. 2007). Commercially available products for CTC detection are based on monoclonal antibodies. Despite their undeniable advantages in the form of high specificity and therefore a low background caused by the lack of binding to antigen-like looking proteins, they recognize only one epitope of antigen. When using such antibodies in different animal species, even small differences in protein sequence can cause lack of binding of such antibody (Lipman et al. 2005). The research on monoclonal antibodies recognizing human blood cells surface antigens showed that only 14% (51 from 380) of them demonstrated a cross-reactivity with canine leukocytes, platelets and erythrocytes (Schuberth et al. 2007). In summary, to determine whether the antibody-based methods have a chance to adopt, it requires the use of empirical research.

Methods based on nucleic acids

Knowledge of the genome organization of a species of interest is required for detailed genetic analyses, including the identification of genes causing hereditary diseases. In the recent years extraordinary progress has been achieved in the dog genome mapping. Moreover, numerous gene mutations causing genetic diseases and a predisposition to develop specific cancers have been characterized and molecular tests for detection of the causative mutations have been developed. It is anticipated that at least 60% of dog diseases have a molecular background similar to that of specific human diseases and are characterized by similar clinical abnormalities (Switonski et al.

2004). The assembled sequence demonstrates that 94% of the dog genome is contained in clear segments of conserved synteny relative to the human and mouse genomes. Out of the 19,000 reported canine genes 14,200 represent 1-1-1 orthologs between dog, human, and mouse (Ostrander and Wayne 2005). It is well known that the process of tumor development results in the accumulation of genetic mutations not only at the level of mutations in individual genes but also in the structure and the number of chromosomes in the cell. Therefore, also the genetic and cytogenetic studies can be used for diagnosis of cancer. It is already known that the mutation responsible for the occurrence of kidney tumors (multifocal renal cystadenocarcinoma and nodular dermatofibrosis) is located in a locus on chromosome 5 (Jonasdottir et al. 2000). Moreover, chronic myeloid leukemia (CML) occurrence in dogs is directly related to translocation similar to that found in humans called the Philadelphia chromosome. Genetic lesions associated with human cancers may be similarly associated with other cancers in animal species (Breen 2009) what means that the cytogenetic examination may be additional method for circulating tumor cells characterization. This use of CTC can give opportunity to observe the karyotype of tumor cells without performing a biopsy. Detection of mutations in genomic DNA can also be a factor in predicting effectivenes of the anti-cancer therapy (Santos et al. 2011) and the data obtained from CTC can be used for this purpose (Leversha et al. 2009).

In veterinary oncology specific mutation responsible for the development of the disease are searched (Chu et al. 1998). Obtained data can be used to find a specific marker for the detection of CTC. Gene expression profile depends on the type and activity of tissue. Using the knowledge on the subject one can select the genes which occur in a selected tissue. This tactics is applied in the case of circulating cancer cells which are of epithelial origin where, as previously described, cytokeratins are used as markers. Until now the usefulness and specificity of selected canine genes in CTC detection has been determined in mammary gland cancer cells from cell culture introduced into the blood. Moreover, some of these genes can also be used in quantitative PCR to predict the level of changes in the tumor (da Costa et al. 2012).

The use of nucleic acids for detection of CTCs in the blood of dogs is the most promising direction in the development of this diagnostic method. An intensive research in these field in human oncology can develop a marker, or set of markers, which will clearly indicate the presence of CTC in the bloodstream. Thus, obtained data can be directly translated to de-tection of these cells in the dog. Databases allow for the genetic human and dog synteny analysis and make easy to find the nucleotide sequence of the desired gene.

Summary

The identification of biomarkers that distinguish diseased individuals from normal is of high interest in many health-related fields. Potential application of biomarkers in veterinary oncology include diagnostic, staging, prognosis and monitoring responses to therapy. The methods and techniques of biomarker discovery and clinical application are often translatable from humans to animals (Henry 2010). So far, there are no existing reports concerning the CTC detection techniques in domestic animals. As demonstrated above, there are possibilities of adapting the circulating tumor cells (CTC) detection methods in veterinary oncology. The introduction of new methods allow for deeper understanding of disease pathogenesis, progression and prognosis, and to develop the effective treatment. It is essential to work on deepening knowledge about circulating tumor cells (CTC) and cancer biomarkers to maximize their positive impact in the field of veterinary oncology.

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