Comparative Medicine
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Vol 64, No 6
December 2014
Pages 502–506

Abstracts of Scientific Papers

AISAL Symposium

Turin, 24–25 October 2014

Main Lectures

Xenotransplantation: State-of-the-Art and Future Perspectives

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Xenotransplantation or transplantation of organ, tissues, or cells between different species—represents one of the avenues explored to overcome the shortage of human donors. In this context, due to physiological, biosafety and ethical reasons, the pig is the donor species which is being studied for a hypothetical future clinical application of this technology. At this stage, there are several obstacles that need to be overcome in order to enable the clinical application of xenotransplantation using porcine organs. These barriers are primarily represented by the immunological incompatibility, some physiological discrepancies and some safety and ethics issues. As far as the immunological incompatibility between pig and primate, recent research has enabled a better comprehension of the processes underlying xenograft rejection. In particular, it has been clarified that the antibody-driven immunity is the one posing the major obstacle. Still, we cannot as yet rule out a contribution of the innate immunity, in particular NK-cells. It is noteworthy that advances in genetic engineering are significantly contributing to the progress of this scientific discipline. In particular, some laboratories have recently engineered pigs lacking expression of some sugar residues and expressing human complement inhibitors. Organs from such pigs survive in non-human primates for 18 months or longer. In the light of the recent progress herewith described, it is expected that further pig engineering will enable the generation of organs that are more compatible and safer to man and will allow further advancement in this difficult scientific discipline.

The Use of the Pig in Pre-clinical and Clinical Xenotranplantation

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The pig is gaining importance in biomedical research as being closer to humans than the mouse and for the increasing restrictions on the use of primates. Pig anatomical and physiological features match very closely those of humans and allow the physician to use the same diagnostic and clinical approaches. Pigs can be easily bred in large numbers under very controlled farming conditions. For these reasons the pig provides both a model for human diseases but also a potential donor of cells, tissues, or organs for transplantation. Pig tissues are already currently used in clinical practice for heart valve bioprosthesis, pig skin for burn patients and now specially treated skin is available for several applications. Pig islet xenotransplantation for treating type I diabetes has entered clinical trials. The use of pig-derived tissues or organs for more complex applications are currently at the preclinical stages of research and the immunological barrier represents the major obstacle as well as the potential risk for zoonosis. To overcome some of these limitations extensive genetic modifications programs have been implemented. Genetic engineering has become more precise and accurate with the use of programmable nucleases. Both the removal of antigenic sugars and the addition of human molecules controlling complement, coagulation and inflammation is providing beneficial experimental evidence in the pig to primate xenotransplantation model. Most of the genetic modifications introduced in the pig genome are well tolerated by the animals and do not impact on their health and welfare.

Tumor Xenografts in Zebrafish Embryo: A Novel Model System in Cancer Research

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The teleost zebrafish (*Danio rerio*) has exceptional utility as a human disease model system and represents a promising alternative platform in cancer research. In particular, when compared to other vertebrate models, zebrafish embryos offer many advantages, including ease of experimentation and drug administration, amenability to in vivo manipulation, and transient and stable

gene inactivation. Also, zebrafish embryo allows disease-driven drug target identification and in vivo validation, thus representing a powerful bioassay tool for small molecule testing and dissection of biological pathways being suitable for high-throughput screening of chemical compounds using robotic platforms. According to current European Union legislation, the use of zebrafish embryos is not regulated and it may help to reduce the impact of animal experimentation by adhering to the 3Rs rule. On these bases, tumor transplantation in zebrafish embryos may represent a simple and rapid approach to study tumor/endothelial cell cross-talk during neovascularization, tumor cell invasion, and metastasis. External fertilization allows the possibility to transplant tumor cells at specific developmental stages and no xenograft rejection occurs at this stage with no need for immune suppressing agents. Many embryos can be injected by a single operator in a few hours improving the validity of statistical analysis. In a few days, tumor cells transplanted into different anatomical sites (e.g. blastodisk, yolk sac, hindbrain ventricle, and bloodstream) can develop tumor masses, providing useful information about the aggressiveness of the disease and the role of specific genes in tumor dissemination and metastasis formation.

Evaluation of Experimental Pulmonary Metastases in Murine Models by X-ray Micro-Computed Tomography

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In vivo lung metastasis platforms are useful to mimic organ-specific microenvironment and biological aspects of clinical cancer, and they are likely to provide relevant pharmacokinetic and pharmacodynamic informations. Several experimental murine models of lung metastases are widely used to test novel therapeutic approaches. Conventional ex vivo analyses require euthanasia at different time points, for performing the macroscopic count and the microscopic evaluation of nodules. X-ray micro-computed tomography (micro-CT) offers high resolution in identifying small metastatic foci in the lungs, and allows a serial monitoring of tumor progression and of therapeutic efficacy in living animals. In this perspective, imaging helps to refine and reduce the use of animals in research, accordingly to the 3Rs principles. We highlight the utility of micro-CT for the in vivo characterization of experimental lung metastases in B16 melanoma and HT1080 fibrosarcoma murine models. A cone-beam micro-CT was used for ungated data acquisition (GE Healthcare Explore Locus, tube voltage: 80 kVp; tube current: 450 uA; number of views: 400; frames averaged: 2; exposure time: 100 ms, pixel size: 45um; scan time: 18 minutes). Images were post-processed using Osirix 5.8.5. A semiautomated region-growing algorithm for quantification of tumor lesions and of areated lung area, as surrogate marker of tumor progression, was used. Volume rendered tridimensional reconstructions were obtained to improve the evaluation of lung metastases pattern. In conclusion, we showed that micro-CT is an accurate and valuable tool to detect and monitor in vivo lung metastases in murine models, and for performing potential preclinical testing of therapeutic efficacy.

Noninvasive Molecular Imaging Techniques: From Ultrasound to Micro-Ultrasound

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Recent developments and improvements of multimodal imaging methods for use in animal research have substantially strengthened the options of in vivo visualization of cancer-related processes over time. Moreover, technological developments in probe synthesis and labeling have resulted in imaging probes with the potential for basic research, as well as for translational and clinical applications. In addition, more sophisticated cancer models are available to address cancer-related research questions. Here we give an overview of developments in these three fields, with a focus on imaging approaches in animal cancer models and how these can help the translation of new therapies into the clinic. Importantly, imaging in living animals helps to reduce the number of animals required per experiment and to provide increased statistical power, as each animal can function as its own control over time. Imaging techniques such as positron emission tomography (PET), single photon emission computed tomography (SPECT), magnetic resonance imaging (MRI), computed tomography (CT), ultrasound, and optical imaging have become dedicated for use in animal models. An important aspect is contrast agents employed during ultrasound and micro-ultrasound experiments. Theranostic nanoparticles enable different imaging probes and therapeutics and contrast agents to be used in one system can therefore integrate multimodal imaging and therapeutic functions, thereby enabling, for example, drug delivery and contrast-enhanced ultrasound. Also, ultrasound has great translational capabilities, as these techniques are already used in the clinic. In this work, we describe and analyze the methods based on ultrasound, micro-ultrasound and photo-acoustic techniques.

About Some Doubts on the Morality of Animal Experimentation

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The issue of animal experimentation is much deeper and wider than usually recognized, because it involves a profound change of world-view. Paradoxically, it is modern science to bring about such a moral change. This becomes clear when we consider that science excludes that in the world there is any grand (purposeful) design putting the *homo sapiens* on the top of creation. In a scientific world view, moral values do not consist in a respectful compliance of an alleged "natural order," but in the (positive or

negative) appreciation that sentient subjects have of the word. We can slightly modify Hamlet's statement and say that "there is no good and bad, but feeling makes it so." Since the most basic moral principle enjoins to prevent or diminish suffering in the world, any sentient being is a "moral patient," worthy of moral consideration. Therefore, the principle of equality should be applied to all sentient beings, in order to avoid speciesism, i.e. the discrimination of someone on the basis of mere belonging to a species. If racism and sexism are morally unacceptable and blameful, because they involve unjust discrimination on the basis of mere belonging to a specific race or to a sex (a mere class of being), so it is species. In this sense, animal experimentation should be carefully reconsidered according to this moral perspective grounded in a scientific world-view.

Xenotransplants, Risks, and Scientific Citizenship

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Xenotransplantation (XT), the transplant of cells, tissues, or organs from animals to humans, has played a paramount and pioneering role amongst emerging technologies in contributing to raise awareness about the impacts of techno-scientific innovation. This unique role has been primarily due to XT safety implications. The potential risks connected to XT, namely the transmission of infections from the animal source to the human recipient and the threats to public health, have been seen as a major reason for citizens' direct involvement in the regulation of XT. Indeed, while different risk models have been adopted by the existing regulations on XT, public involvement has become a widely accepted international criterion and indicator for legitimate clinical experimentation in XT, also recognized by WHO. This contribution explores the meaning, the rationale, and the evolution of the role of society in XT, from the notion of public consultation to the framing of rights of scientific citizenship. It also analyzes how these rights are acquiring momentum not only in relation to the social acceptability of technological risks but, at least in perspective within the European Union regulation, also as to the very use of animals for scientific purposes.

Transgenic Mouse Models for Cancer Research: The Example of BALB-NEUT Mice

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The predictive utility of tumor models depends on the fidelity with which they recapitulate the heterotypic interactions between the incipient tumor and the immune system, the endothelial cells, the tumor-associated fibroblast, and additional stromal components that take place during tumor progression. Genetically modified mice (GEM) engineered to express oncogenes, or in which tumor suppressors have been disrupted, and that spontaneously develop tumors provide a good step forward in this direction. The relationships between the incipient tumor and the surrounding tissues are preserved, while the progression of carcinogenesis may mimic what is observed in human. Indeed, the advent of GEM has revolutionized preclinical cancer research, and several successful preclinical results in different GEM models have been reached. BALB/c mice transgenic for the transforming form of the rat Her2/neu oncogene under the transcriptional control of the MMTV promoter (BALB-neuT mice) are a good example of how GEM can impact cancer research. BALB-neuT mice spontaneously develop mammary carcinomas with 100% penetrance and display a histopathologically and transcriptionally well-characterized course that closely recapitulates many features of human breast carcinogenesis. These mice have provided us with a fascinating tool and one that is used in many laboratories worldwide to deepen current knowledge of the pathogenic mechanisms that promote Her2 positive tumor growth and consequently elaborate more efficacious antitumor strategies.

Xenograft Models for the Analysis of New Therapeutic Approaches

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P53 is one of the most relevant human oncosuppressor genes. Accordingly, inactivation of p53 is the most frequent lesion in human tumours. One important event leading to p53 inactivation is its binding to the heterodimer formed by the protein inhibitors, MDM2 and MDM4. Many anti-cancer therapeutic approaches have been explored in order to release p53 from its inhibitors and re-establish its oncosuppressive activity. Particularly, different molecules able to bind MDM2 or MDM4 allowing the release of p53, have been isolated and analysed. Since these molecules target the human proteins, in vivo test of their efficacy is based on the utilization of xenograft models consisting of human tumor cells in nude mice. Depending on the properties of these molecules (solubility, stability, etc.), subcutaneous or orthotopic xenografts have been developed. Recently, we have developed a peptide able to dissociate MDM2 from MDM4 and impair the inhibiting activity of the heterodimer towards p53. The in vivo test has been carried out by injecting the peptide intratumorally in a subcutaneous xenograft model. In order to reduce the number of mice for in vivo test, we have taken advantage of luciferase-expressing engineered human tumor cells. These experiments have allowed in vivo confirmation of the efficacy of the peptide and to foster further the research on it.

Measuring Mechanical and Thermal Sensitivity in Healthy and Diabetic Mice Lacking Ghrelin

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Ghrelin exerts antinociceptive effects by mitigating inflammatory reactions and modulating neuronal activity in the spinal dorsal horn. We investigated the role of ghrelin and its receptor GHSR1a in modulating sensory behavior in healthy and diabetic mice, and tested the hypothesis that ghrelin's effect on sensory phenotype is associated with spinal microglia. Four-week-old male C57BL/6 mice (WT) and double knockout (dKO) mice lacking both ghrelin and GHSR1a received single intraperitoneal injection of citrate buffer or streptozotocin (STZ) to induce diabetes. Mechanical threshold was measured by von Frey hairs and thermal sensitivity was measured by the tail immersion test weekly. Animals were sacrificed at 8 postnatal weeks, and spinal microglia stained with anti-Iba1 antibody. At 4 weeks, dKO mice showed an increased mechanical and thermal sensitivity compared to WT. However, while differences in thermal sensitivity disappeared with development, those in mechanical threshold increased. Interestingly, Iba1 stained a significantly smaller area in dKO spinal dorsal horn than in WT, implying an altered microglial phenotype. We asked whether such a difference was relevant for the development of pain hypersensitivity in diabetes. STZ-WT mice developed a strong hyperglycaemia associated with increased mechanical sensitivity. Surprisingly, STZ-dKO were more resistant to developing both hyperglycaemia and mechanical hypersensitivity. Our data support an antinociceptive role of ghrelin, which might involve microglia-neuron communication in the spinal dorsal horn. However, ablating ghrelin signaling does not worsen diabetes-induced hypersensitivity as it delays the onset of hyperglycemia as well as diabetes-associated alterations in nociceptive behavior.

A Xenograft Model Highlights the Dichotomic Effects Induced by Adiponectin on Cyclin D1 in Human Breast Cancer Cells Accordingly to ERA Expression

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Adiponectin is a peptide hormone secreted exclusively from adipose tissue and exhibiting plasma levels inversely correlated with BMI. Low levels of plasma adiponectin appears to be associated with a mayor tumor growth and invasiveness for obesity-related cancers such as colon, prostate, endometrial, and breast cancer. However, a controversial issue concerns the role of adiponectin on breast cancer cell (BCC) growth. Our results obtained using two different experimental approaches, an in vitro and in vivo xenograft model display a dichotomic effects induced by adiponectin on BCC growth. In animal studies we showed that adiponectin significantly reduces tumor volume in mice implanted with human ER-negative MDA-MB-231 cells, while the opposite effect was observed in mice implanted with human ER-positive MCF-7 cells. Then, we found that in MCF-7 cells adiponectin induces the up-regulation of cyclin D1 (CD1), at mRNA and protein levels, while in MDA-MB-231 down regulates its levels. Transient transfection experiments and site-directed mutagenesis assay confirmed the dichotomic effects of adiponectin on CD1 promoter, identifying the Sp1 motif as a putative site of regulation of CD1 promoter activity. In ER-negative BCC, adiponectin through the recruitment of a corepressor complex, inhibits CD1 expression and arrests breast tumor growth. In contrast, in ER-positive BCC, adiponectin recruits an activator complex which increases CD1 expression, inducing breast tumor growth. We may conclude that adiponectin, in the range of the physiological concentrations tested (1and 5µg/ml), modulates the CD1 activity in BCC growth and progression and these effects are tightly dependent on Estrogen Receptor alpha (ER) expression and its signaling.

The hSOD1-G93A Transgenic Swine as a Potential Animal Model for Amyotrophic Lateral Sclerosis

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Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease that occurs in two forms: sporadic and familial, the latter linked to mutations in the SOD1 gene. The use of mice carrying the hSOD1^{G93A} mutation is currently widespread in ALS research; however a real improvement of patient prognosis has not yet been obtained. Another model, more closely related to human species, is strongly demanded by the scientific community that has already foreseen swine as an attractive alternative for modeling human neurodegenerative diseases. Recently we produced four hSOD1^{G93A} cloned boars that were analysed to confirm the transgene integration and expression, while its long-term effects are still under investigation. In order to assess if this species may represent a suitable model in reproducing ALS features, an extensive phenotypical characterization, was applied. Clinical and neurological examination protocols were applied. Motor function and gait dynamics were evaluated using an integrated protocol of digital gait analysis (3D Motion Capture) and surface electromyography (EMG). Furthermore, tissues from transgenic and age matched control piglets were analyzed by immunohistochemistry and immunofluorescence. Immunohistochemistry demonstrated granular mutant protein aggregates in the brain and spinal cord. Since this is the first swine ALS model produced so far, further investigations are required to determine disease onset, duration, and the pathological features. Thus our aim is to reach an exhaustive characterization, in the hope that possible positive outcomes obtained in the swine species could be transferable to ALS patients. Support GR-2010-2312522; IZSPLV 04/10RC

Use of Permethrin Treatment on Genetically Modified Mice Housed in Isolators Infested by Fur Mites (*Myobia musculi*)

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Fur mite infestations are common in many rodent facilities. Obtaining complete eradication of a fur mite infestation is often difficult to accomplish. We investigated the effect of a permethrin treatment on a natural infestation by *Myobia musculi* of 24 different genetically modified mouse colonies housed in positive pressure plastic film isolators. It had been previously documented that permethrin is effective to eradicate mite infestations in combination with an environmental decontamination (permethrin indeed is not ovicidal). We assessed therefore the treatment of a large colony containing 2,250 genetically modified mice housed in 9 plastic film isolators by using only cotton balls bedding impregnated with 7.4% permethrin solution. A total of 3 cotton balls per adult rodent and 1 cotton ball for each pup were placed in all cages as nesting material and replaced weekly at cage cleaning. After 6 weeks treatment, a period of two weeks without treatment was provided. This was then followed by and additional administration period of 3 weeks. At the end of the treatment, health monitoring was conducted on both original animals and dirty bedding sentinels. All animals remained mite-free to date (11 months, N=518 tested mice). To our knowledge this study is the first analyzing the efficacy and the feasibility of a treatment against mites on mice housed in isolators. In our experience this treatment is free from adverse effects, reliable, and can be considered as a valid option for eradication of fur mites in environments difficult to decontaminate.

Colorectal Cancer Xenopatients as a Preclinical Platform for Precision Medicine in Metastatic Colorectal Cancer: Opportunities and Challenges

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Preclinical validation of potential therapeutic targets using in vivo models is traditionally regarded as an obligatory step of anticancer drug development, but it is also considered a problematic issue. There is now rising concern that what is still deemed a successful endpoint at the preclinical level, i.e. positive performance of a drug in xenografts of different human cancer cell lines, is in fact not predictive of compound efficacy in the clinic. The obvious objection is that immortalized cancer cells exhibit a genetic drift and phenotypic features different from original cancers in patients. Another drawback of such an approach is that the catalog of currently available cell lines is inevitably finite. Therefore, experiments with cell lines cannot recapitulate the wide heterogeneity of human malignancy that occurs among individuals on a population basis. One robust way to proceed with efficient, high-fidelity drug development at the stage of in vivo validation, while minimizing the effects of uncharacterized tumor heterogeneity, would be to perform preclinical population-based studies. To do this, we implemented a biobank of patient-derived surgical samples stored under viable conditions and systematically passaged in mice, with a focus on colorectal cancer. Serial mouse expansion leads to the generation of treatment cohorts that can be concomitantly profiled for deep molecular analysis and subjected to treatment with investigational therapies. This platform ensures reliable execution of genotype/response correlations for high-fidelity anticipation of clinical findings and allows derivation of viable tumor material for genetic manipulation and mechanistic exploration.

Electrophysiological Characterization of Painful Peripheral Neuropathy Induced by Bortezomib in Rats

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Bortezomib (BTZ) is an antineoplastic drug mainly used for the treatment of multiple myeloma and some types of solid tumors. Despite its effectiveness, BTZ clinical use is frequently limited by the onset of painful peripheral neuropathy, generally associated with alterations of A delta and C type primary afferent fibers. The neurotoxic mechanisms of BTZ remain poorly understood, although proteotoxic stress and mitochondrial impairment may contribute to the pathogenesis of the sensory distal neuropathy. To evaluate these aspects, spinal cord electrophysiological recordings and behavioral test alterations for neuropathic pain and immunohistochemistry studies of pain-related sensory biomarkers were performed in a Wistar rat model of BTZ-induced painful peripheral neuropathy. Then, three different analgesic drugs (CR4056, gabapentin, and buprenorphine) were tested against BTZ-painful neuropathy. The animals were intravenously treated with BTZ 0.20 mg/kg, 3 times a week for 8 weeks, followed by 2 weeks of analgesic administrations. Bortezomib induced both mechanical allodynia and peripheral neuropathy in rats after 8 weeks of treatment. CR4056 was able to recover the painful condition without changing the nerve structural damage. Moreover, BTZ- induced changes both in the electrical activity of wide dynamic range neurons, and in the transient receptor potential vanilloid type 1 receptor (TRPV1) and neuropeptides calcitonin gene-related peptide (CGRP) immunolabeling expression in the spinal cord. In conclusion, the results of the study suggest that the alteration in the spinal cord electrophysiological recordings and CGRP/TRPV1 levels modulation are involved in the persistence pain symptoms in BTZ-induced peripheral neuropathy. Therefore, this model will enable us to test promising analgesic drugs designed to ameliorate or restore painful condition, and to compare it with standard compounds. Supported by AISAL.