THE COLLABORATION BETWEEN UNIMARCONI AND ENEA: MULTIDISCIPLINARY ASPECTS

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On November 18, 2014, the University Guglielmo Marconi held a half-day symposium marking the first five years of collaboration with the ENEA’s Laboratory of Radiation Biology and Biomedicine. The title of the event was: “The Collaboration between Unimarconi and ENEA: Multidisciplinary aspects”. This paper gives a summary of the scientific presentations.

Biodistribution and toxicity in vivo after intravenous administration of nanoparticles (P. Giardullo)

“Nanomaterials” is a term typically used to describe discrete pieces of matter with a critical diameter between 1 and 100 nm. Nanoparticles have a number of properties that distinguish them from bulk materials simply by virtue of their size, such as chemical reactivity, energy absorption, and biological mobility. This is typically because nanoparticles have a greater surface area per weight than larger particles which causes them to be more reactive to some other molecules.

Nanoparticles are used, or being evaluated for use, in many fields: manufacturing and materials, environment, energy and electronics and in recent years, there has been an unprecedented expansion in the field of medicine, with applications ranging from contrast agents in medical imaging to carriers for drug delivery and to targeted therapeutic applications. Nanoparticles are characterized by high stability, high carrier capacity, the ability to incorporate both hydrophilic and hydrophobic substances and compatibility with different administration routes, thereby making them highly attractive in many aspects of medicine, in particular of oncology (Avnesh et al., 2013). Drug delivery is one of the major areas in which nanotechnology is helping revolutionize the treatment of cancer. The drug can either be adsorbed, dissolved, or dispersed throughout the nanoparticle complex or, alternatively, it can be covalently attached to the surface. Some nanoparticles used
to treat cancer have either been approved or are still in clinical development. For example, Doxil is a PEGylated liposome loaded with doxorubicin that has been approved by the US Food and Drug Administration (FDA) for the treatment of patients with ovarian and metastatic breast cancers and human immunodeficiency virus-related Kaposi sarcoma.

Nanoparticles have shown great promise as molecular imaging agents to detect and monitor cancer progression. For example, when using optical imaging modalities, nanoparticles can increase signal intensity, thereby allowing fewer numbers of cells to be imaged at greater tissue depths, as well as providing imaging signals that are stable over longer periods of time.

Nanoparticles are able, also, to increase their therapeutic index. For example, they can function as therapeutic agents in photodynamic and thermal therapy. The nanoparticles used in photodynamic therapy can deliver light-activatable chemicals, known as photosensitizer molecules, to tumor cells. After the absorption of light, photosensitizer molecules can generate cytotoxic oxygen-based reactive species, which can subsequently cause cellular damage and cell death via oxidative stress. Photothermal therapy (PTT) uses photothermal agents to achieve more controlled and selective heating of the target area, thereby confining thermal damage to the tumor. Nanoparticles can be used in PTT to cause localized destruction of tumors after absorption of light due to their efficient light-to-heat conversion.

The emerging research field in nanomedicine, is to develop a “smart nanoparticle” that can diagnose, deliver targeted therapy and monitor the response to therapy in a single integrated system. This concept is known as theranostic nanomedicine that is an important ongoing challenge of the cancer treatment.

Nanoparticles have unique physico-chemical features that facilitate novel applications, but on the other hand, the same nano-scale properties can potentially induce cytotoxicity that can manifest itself by impairing the functions of the major components of the cell (Neenu et al., 2010). Although several studies have investigated the toxicity associated with specific nanoparticles, the results are highly variable. Hence, short-term and long-term toxicity studies will also need to be undertaken in both cell culture and living animal models before they can gain approval for clinical trials. In this context, a recent paper was published in the International Journal.
of Nanomedicine, resulting from a multidisciplinary collaboration between the Guglielmo Marconi University and different ENEA’s laboratories (Bellusci et al., 2014). Here, we investigated the uptake, in vivo biodistribution, and in vitro and in vivo potential toxicity of manganese ferrite (MnFe$_2$O$_4$) nanoparticles. MnFe$_2$O$_4$ is a supermagnetic iron oxide nanoparticles (SPION) that has immense potential in a variety of biomedical applications.

For the experiments, cultures of murine Balb/3T3 fibroblasts were exposed for 24, 48 or 72 hours to increasing ferrofluid concentrations. The in vitro study demonstrated dose-dependent nanoparticle uptake and statistically significant cytotoxic effects. The nanoparticle biodistribution and clearance was also evaluated in mice, after a single intravenous injection in vivo, by Mn spectrophotometric determination and Prussian Blue staining in liver, kidneys, spleen and brain at different post-treatment times up to 21 days. No evidence of histopathological irreversible damage to any of the tested organs was observed. A comparison of the lowest in vitro toxic concentration with the intravenously injected dose and the administered dose of other ferrofluid drugs currently in the clinical practice, suggests that there might be sufficient safety margins for further development of this ferrofluid formulation.

From these important results, a new collaboration was established to study the use of plant virus nanoparticles, which appears very intriguing for the wide diversity they offer concerning symmetry and dimensions and the ease of chemical and biological engineering of both the surface and/or the internal cavity. Moreover, these self-assembling nanostructures can be produced in plants easily, safely and rapidly, and might represent an ideal delivery tool in terms of biocompatibility and biodegradability.

This emerging field offers the possibility of developing new therapeutic tools, combining nanotechnology with agents directed towards specific signaling pathways involved in tumor formation.
Abscopal radiation effects
(M. Mancuso and A. Saran)

Partial body irradiation exposures are the norm rather than the exception in radiation therapy, imaging and many workplace exposures. Work at ENEA has provided the first proof-of-principle that brain cancer in genetically sensitive mice is increased by exposure of distant tissues, indicating that there is a level of communication between irradiated and non-irradiated tissues (Mancuso et al., 2008).

In the framework of the collaboration between Unimarconi and ENEA, we further explored the mechanisms behind radiation abscopal responses with a view to providing mechanistic insights into dose and spatial effects in long-range radiation signaling in mammals. We again used the shielded cerebellum of radiosensitive Patched1 heterozygous knockout (Ptch1+/−) mice after partial-body X-ray exposure as a model system.

Because most environmental, occupational, and medical exposures occur at low doses, it is important to estimate the influence of lower dose levels on abscopal phenomena. We therefore tested the dose dependence of long-range bystander signaling in vivo using doses of 1, 2, or 3 Gy by whole-body (WB) irradiation protocols and after shielded (SH) irradiation. For cellular short-term effects (i.e., apoptosis) in shielded cerebellum tissue we observed clear dependence on dose to the irradiated body, suggesting that, under in vivo conditions, increasing radiation dose results in increased probability of triggering long-range apoptotic effects in off-target CNS tissue. In contrast, the pattern of abscopally induced CNS tumors indicated both the possibility of a lower activating threshold for tumorigenic damage and an all-or-nothing nature of the abscopal tumor response. In fact, the extent of the effect at 2 Gy was virtually identical to that measured at 3 Gy (Mancuso et al., 2008), with tumor rates significantly higher than in control mice (P < 0.05).

Conversely, no excess brain tumor mortality was found in SH mice irradiated with 1 Gy over control mice. These data suggest that whereas some of the out-of-field biological responses may increase with increasing dose, other effects in unirradiated tissue are induced in a binary fashion (Mancuso et al., 2012).

Moreover, in order to establish whether the range over which the abscopal signal has to propagate, and/or the amount of directly
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irradiated tissue were able to modify abscopal responses in the brain, $Ptc1^{+/−}$ mice were treated in a variety of partial-body irradiation protocols, which allowed to vary the proportion of the mouse body in and out of the irradiation field. Shields were designed to cover ∼one third of the mouse body (i.e., head and shoulders; Mancuso et al., 2008), or two thirds (by means of longer single shields, or two shields placed on the head and caudal part). We used doses of 3 and 10 Gy. This experiment showed that the proportion of the mouse body in and out of the irradiation field is crucial because this may have a direct impact on signal effectiveness: when mice were irradiated (3 Gy) with shields of different geometry, however covering two thirds - instead of one-third - of the body, a sparing of apoptosis was observed, and this effect was associated with abrogation of abscopal tumorigenesis. The decrease in apoptotic cell death and lack of oncogenic effects by use of larger shielding was also not influenced by the distance factor, suggesting a space independent phenomenon. This is consistent with in vitro data showing damage in unirradiated bystander cells far away from the irradiated areas (Hu et al., 2006) and with results of microbeam experiments showing a relatively long range of apoptotic bystander signals in human artificial tissue (Belyakov et al., 2005).

We then asked whether a very high radiation dose would even out the differences in biological responses in out-of-field cerebellum at varying distance from irradiated areas. The data obtained from analysis of shielded cerebellum tissue in mice irradiated with 10 Gy showed that increasing the dose results in increased signal strength for the apoptotic endpoint and that a high dose to the exposed body can compensate the decreased amount of mouse body exposure in terms of carcinogenic risk in unexposed brain. Therefore, by exploiting the dose- and volume dependent properties of the system, distant bystander signals can be increased in their power - enhancing the magnitude of effects in abscopal regions - not in dynamic range (Mancuso et al., 2012).

Understanding the biological effects occurring in unexposed tissues after partial-body irradiation is relevant in the radiation therapy context because it may help potentiate radiation therapeutic efficacy and clarify the collateral risks to normal tissues remote from the tumor target.
Non-cancer effects of ionizing radiations: animal models for the study of the mechanisms (S. Pazzaglia)

In addition to cancer, ionizing radiation exposure can induce non-cancer effects. These effects occur when cell death is so extensive to cause functional impairment of the tissue or organ. The main non-cancer effects are cardiovascular disease, neurocognitive effects and lens opacities. It has been traditionally assumed that health effects other than cancer show a threshold at doses that are well above the levels of exposures typically encountered in the public environment, at work, or from medical uses of ionizing radiation. Recent results from epidemiological and experimental studies indicate increased risks from cardiovascular diseases, cataracts, and cognitive effects not only at doses above 5 Gy but also in a range of doses from 5 to 0.5 Gy and, possibly even at lower doses (<0.5 Gy). In 2011, the International Commission on Radiation Protection (ICRP) recognized a new dose limit of 0.5 Gy for the lens of the eye, and a recommendation was made for a reduction in the annual absorbed dose limit to 20mSv. The mechanisms responsible for the non-carcinogenic effects of radiation are for the most part unknown. Research to understand biological mechanisms underlying radiation-related non-cancer effects is necessary and animal models may help identifying these mechanisms. Examples of experimental mouse studies on cardiovascular diseases, cognitive effects and cataract are provided below.

Neurocognitive effects

Neurological deficits including impairment in memory, attention and executive function are frequent after high-dose cranial irradiation for radiotherapy with effects more pronounced in children than in adults. The increasing use of radiation procedures such as computed tomography (CT) scans raised concerns on potential harmful effects of low radiation doses to the brain in the general population, and especially in children. To assess long-term cellular and molecular alterations induced by low-dose irradiation, newborn mice (NMRI) of 10 days of age were exposed to 20, 100, 500, or 1000 mGy of γ-Rays. Six months later, they were subjected to cognitive evaluation and immunohistochemical analysis of the dentate gyrus (DG) of the hippocampus to stage neuronal differentiation and to
identify modification of the microenvironment. We focused on the hippocampus because the learning memory and spatial information processing abilities are dependent on proper hippocampus functionality, and because the DG is one of the two structures of the central nervous system where continuous neurogenesis is observed throughout life. Our analyses revealed a strong association between cognitive dysfunction, impaired neurogenesis and neuroinflammation in the hippocampus after irradiation at low/moderate doses. No single cell type alteration can fully explain the complexity of the long-term consequences of irradiation. A dynamic interaction between multiple cell types (i.e., neurons, microglia and astrocytes) is probably involved in the pathogenesis of radiation-induced cognitive injury.

**Cardiovascular diseases**

Radiation is a risk factor in human vascular disease and an increased incidence of atherogenesis is observed in patients with Hodgkin’s disease, breast cancer and head and neck cancer after radiotherapy. The apolipoprotein E-deficient (ApoE<sup>-/-</sup>) mice, exhibiting a marked increase in the levels of plasma cholesterol when fed a normal chow diet (4.2% fat), are predisposed to spontaneous atherogenesis and develop lesions resembling those seen in humans. As a consequence, they represent an ideal mouse model to establish the role of ionizing radiation in the atherogenic process in vivo. To evaluate cardiovascular alterations, aortas from irradiated ApoE<sup>-/-</sup> mice were subjected to morphometric and histological analyses. We found that acute exposure of ApoE<sup>-/-</sup> mice to high (6 Gy) or low (0.3 Gy) radiation doses enhanced atherogenesis by increasing the percentage of aortic area covered by plaques. Interestingly, irradiation failed to cause an increase in plaque size, yet causing increase in plaques density. This suggests that radiations act mainly as an initiating stimulus for atherosclerotic plaque development.

Histopathologic and vascular studies show that plaque composition and vulnerability (type of lesion) are crucial factors that may lead to sudden rupture of the plaque surface, underlying the great majority of infarctions. As markers of vulnerability of the plaque we examined, through immunostaining, the thickness of the fibrous cap (SMA) and the inflammation index (CD68). We observed an increase in percentage of total plaque area occupied by macrophages (CD68<sup>+</sup> cells) and decrease of smooth muscle cells (SMA<sup>+</sup>), indicating a
thinning of fibrous cap after irradiation with 6 Gy. A thinning of the fibrous cap decreases lesion stability and is a typical feature of advanced lesions.

**Lens opacities**

Cataract (the opacification of the ocular lens) is the most frequent cause of blindness worldwide. The eye is well known as one of the most radiosensitive body-tissues and it is clearly recognized that cataracts can be induced by ionizing radiation exposure. We investigated the mechanisms of radiation-induced cataract development in $\text{Ptch}^+-/-$ mice, a well-known mouse model of radiation-induced oncogenesis. Irradiation of $\text{Ptch}^+-/-$ and wild-type mice with 3Gy of X-ray at postnatal day 2 (P2), a very early stage of lens development, significantly increased cataract development compared to unirradiated $\text{Ptch}^+-/-$ mice. Instead, irrespective of $\text{Ptch}^1$ status, very low or no induction of cataracts was observed when radiation was delivered at P10, when lens development is near to completion, or at P56, when development is completed. These results indicate a clear age-related window of susceptibility in cataract induction by ionizing radiations. In addition, these data highlight a novel function of Sonic Hedgehog (Shh) signaling unrelated to cancer, and provide a new relevant animal model to investigate the molecular pathogenesis of cataract formation.

**Conclusions**

The use of mouse models may be helpful for identifying the biological and molecular mechanisms by which radiation exposure increases non-cancer disease risks. A better understanding of the mechanisms of non-cancer effects at low/moderate radiation doses is critical in establishing health risks and protection-related policies.

**Gender effects in preclinical models of human medulloblastoma: role of estrogen receptor $\beta$**

(I. De Stefano)

Estrogens constitute a class of steroid compounds traditionally associated with female reproduction. The last decade has seen a revolution in our understanding of the actions of estrogen in the body. Estrogens play important roles in both reproductive and non-
reproductive systems. They can be synthesized in non-reproductive tissues such as liver, heart, muscle, bone and brain. Very recent data demonstrate a crucial involvement of estrogen signaling in carcinogenesis of non-classical estrogen target tissues (e.g., brain, skin, colon, prostate and lung).

The biological effects of estrogens are mediated by two distinct estrogen receptors (ERs), ER\(\alpha\) and ER\(\beta\). Although ER\(\alpha\) and ER\(\beta\) have similar structures, they mediate different effects, and there is currently increasing evidence that an imbalanced ER\(\beta\) expression might play a pivotal role in development and progression of some tumors (Gallo et al., 2012).

The brain is now recognized to be affected by estrogen, in particular both ER\(\alpha\) and ER\(\beta\) are expressed in the cerebellum. Current literature data suggest that endogenous/exogenous estrogen exert a protective role against the development and progression of some brain tumors (e.g., glioma, medulloblastoma [MB]), while may be detrimental in other tumor types (e.g. meningioma).

MB, a highly malignant primitive neuroectodermal tumor of the cerebellum, represents about 20% of all childhood primary CNS tumors. The peak age at presentation is 3-6 years, with only 25% of patients between 15 and 44 years of age. Epidemiological studies have shown that male sex is a risk factor for MB, irrespective of age, race or region of the world with approximately 65% of patients being males. Besides this different susceptibility, female gender is also a significant favorable prognostic factor in MB, with girls having a much better outcome.

In line with these epidemiological findings, using the well-characterized \(Ptch1^{+/}\) mouse model of radiation-induced MB, we previously showed a protective action of estrogen during early stages of MB development; indeed, susceptibility to MB development was significantly increased in ovariectomized \(Ptch1^{+/}\) females, and restored to levels observed in control mice after estrogen replacement. We next investigated the molecular mechanisms by which estrogen might influence tumor progression, and showed that ER\(\beta\), but not ER\(\alpha\), is involved in modulation of MB development by estrogens. These effects were achieved via activation of anti-proliferative and pro-apoptotic pathways (Mancuso et al., 2010, Mancuso et al., 2011). In this context, we recently published a paper in PlosOne, in collaboration with the Catholic University of Sacred Heart in Rome, which evaluated the impact of gender and the role
of ERβ in preclinical models of human MB. We found that the growth of D283Med human cell line boy, xenotransplanted in male and female nude mice, was strongly dependent on gender. Indeed, tumors growing in females were significantly smaller compared to those growing in males. Moreover, at microscopic examination, tumors from females showed a shift towards differentiation, as evaluated by a decreased stem cell population component (evidenced by expression of the stem-like cell marker for MB, nestin), and a substantial increase of neuronal and glial differentiation markers.

Hormone receptor expression profile in tumors from both female and male mice showed that the expression of ERβ isoforms was differentially regulated in tumors from males and females. Indeed, while ERβ2 and ERβ5 were consistently expressed in all tumors independently of gender, ERβ1 expression was significantly higher in tumors from females compared to males.

In summary, we have shown a significant sex effect on MB growth in a preclinical mouse model of the human disease, reflecting the greater prevalence of MB in males compared to females in the human population. We have also provided mechanistic evidence supporting the idea that ERβ1 signaling may have pro-differentiation and tumor suppressive functions in MB, thus suggesting that functional activation of the ERβ pathways may be a potential therapeutic option for MB (Ciucci et al., 2014).

References


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