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THE 3RS IN TUMOR MICRO-ENVIRONEMENT STUDIES: EMPIRICAL BASES OF THE ETHICAL REGULATION

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Abstract

With the general goal to provide a rationale for the application of the 3Rs rule (Replace, Reduce, Refine), we aim to study the effect of the tumor microenvironment (TME) on the tumor response to a chemotherapeutic agent and use this research as a series of case studies in which to assess the application of the 3Rs rule from an epistemological point of view. In particular, by comparing reiterated experiments using 2D and 3D tumor cell cultures with murine models of cancer, we aim to assess to which extent mice can be **R**eplaced or their number **R**educed. In addition, we propose to further characterize a novel, **R**efined model of cancer that better mimics humans in respect to classical murine models.

Keywords: 3R, tumor, ethical studies

The relevance of the tumor microenvironment (TME) for both clinical and mechanistic, biological studies on tumor pathophysiology is now established, albeit being a recent achievement. Indeed, the behavior of tumors and their interaction with the host, ranging from immune response to cancer-induced cachexia, are so deeply affected by the TME that current studies on cancer progression and response to chemotherapy cannot ignore this issue any longer. While it is obvious that tumor cells are within a three-dimensional environment in living organisms, recreating the correct tissue architecture *in vitro* to mimic the TME is not a straightforward endeavor. Since there are noteworthy limitations with two-dimensional cell cultures experiments, a major effort has been done toward the creation of 3D cultures, as they allow to understand how microenvironmental cues affect tumor biology (Hutmacher 2014). 3D constructs typically include extracellular matrix (ECM, Senthebane 2018), stromal cells (Vickman 2020), and/or immune cells (Di Modugno 2019) in such a way that goes far beyond standard cocultures approaches. The rationale for the *in vitro* approaches to study tumor biology stems from the regulations of animal experimentation (directive 2010/63/EU On the protection of animals used for scientific purposes) that require any experimental plan to undergo the review by an ethical committee, before being approved by the Ministry of Research: a mandatory requirement to pass this peer review is to follow the 3Rs rule – namely Replacement, Reduction, Refinement - which indirectly encourages *in vitro* approaches, as better detailed below. Indeed, current guidelines recommend that researchers dealing with animal experimentation must wonder whether and by what alternative setup the animals they plan to use might be replaced, whether and how their number can be reduced, and whether and how animal experimentation might be refined, i.e. transformed in such a way as to obtain better information with a lower number animals. This process either results in the use of *in vitro* models that are used in preliminary studies and even in the replacement of the animal models or it culminates with the argumentation that animals are essential for a specific research project and cannot be replaced. More fundamentally, the ethical advice given by ethical committees is based on a rather weird cost-benefit analysis. Cost is assessed on ethical grounds: the number and the well-being of animal models are the currency for the computation; benefit, on the other hand, is assessed in epistemic terms, namely, in terms of resulting knowledge. However, ethical and epistemic currencies are difficult to compare; importantly, the resulting knowledge is only assessed, in the published research papers, based

on the research project as defined by the researchers, not on whether the 3Rs have been complied with. There is, thus, a gap between the way scientific knowledge results from the research process, on the one hand, and the ethical features of the process, when it comes to animal experimentation, on the other hand. How can this gap be filled? Probably by re-connecting more closely the ethical side of animal experimentation, based on compliance with the 3Rs, with the scientific goals of the researchers using animal experimentation. This may be done by relying on the growing literature analyzing the epistemological aspects of the use of animal models in animal experimentation (Ankeny & Leonelli 2011, Baetu 2016, Burian, 1992, 1993, Geison & Creager 1999, Leonelli & Ankeny 2012, Levy & Curie 2015, Weber 2014).

MATERIAL AND METHODS

Within TME and cancer cachexia research, the justification that animal experimentation cannot be replaced is usually based on the fact that *in vitro* experimentation on cell cultures is unable to capture the holistic feature of the interactions (I) within TME and (ii) of TME with other systems or organs, like muscles. Cell culture indeed does not seem adequate to investigate the complexity of these interactions. However, the latter idea is usually *assumed* rather that demonstrated: it represents, therefore, a prejudice. With the goal to provide a rationale for the application of the 3Rs rule, we had five specific aims. The biological part will aim to assess whether and to what extent the 3Rs can be applied to the context of TME, chemotherapy and cancer cachexia. The philosophical branch of the project analyzed the methods used to compute the number of animals required to test a given hypothesis and, *a posteriori*, the results obtained with the experimentation from the biological branch; in addition, the philosophical branch will address the issue of the correct way to formulate hypotheses for projects dealing with TME and cancer cachexia studies. The experimental models used in this study consisted, for the biomedical part, in 2D and 3D C26-tumor cell cultures, as well as in tumor-bearing mice (BALB/c mice subcutaneously grafted with the C26 colon carcinoma and the KPP mice, which represent an inducible model of pancreatic ductal adenocarcinoma). The fact that tumor-bearing mice are already in use in the laboratory for studies on cancer-induced muscle wasting will avoid adding up a significant amount of extra animals to the experimental plan, since the analyses of these mice will simply be extended to the tumor mass in addition to the musculature.

The aim of the project was to investigate on the reproducibility of the murine models by cell culture models, which requires to drive conclusions from the comparison of results issuing from very different models; to do so, each set of data obtained from a given experiment was analyzed by comparison with its inner control (e.g. non-treated population) and expressed as fold-induction; negative controls was provided along with the positive controls, represented by some well-known outputs, such as the cytotoxic effects of chemotherapy on tumor cells (see below for further details). Thus, first the sets of data coming from the experimental groups was compared with each other, secondly the “behavior” of the data in different experimental models will be rivalled, and ultimately conclusions will be drawn based on the latter analysis. The epistemological analyses was rely on the experimental process in its integrity and will differentiate the kinds of reasoning involved at each step: comparison with inner control, with negative controls, among groups; construction of overall conclusions; assessment of the validity of the whole process. It is important to pay attention to involved inferences, because, in spite of being blinded, so to speak, in ethical agreement forms, they impact the way the 3Rs are complied with.

RESULTS AND DISCUSSIONS

The cytostatic and cytotoxic effects of cisplatin on C26 tumor cells are known and expected. However, we expect that the TME will affect the C26 cell response to chemotherapy and that the comparison of *in vitro* and *in vivo* results will show significant differences. This will say if, which one, and to which extent an *in vitro* model can replace the use of the mice. All of the above will represent an empirical proof of principle on the validity of the Replace principle. While performing and analyzing experiments in a reiterative way along the project, we expect the statistical significancy level to reach a plateau, indicating the existence of a minimum number of replicates necessary to demonstrate an effect (reduction of the number of experiments to a

minimum). We also expect that the different experimental models will reach this plateau in different moments, suggesting that one approach is more sensitive (refined) that others for this specific test. This task, also dealing with refinement, will complete the characterization of a novel animal model of cancer, in which the TME is closer to the clinical practice, being the tumor orthotopically and not ectopically localized and determining host (mouse) responses closer to humans: in particular the functional studies will show how cachexia drives the loss of skeletal muscle force and the increase in fatigue in this animal model. By comparing what researchers say in ethical agreement forms about animal experimentation and what kind of research results they obtain therefrom, we discovered ways to improve the mobilization of the researchers' knowledge about model animals in their implementation of the 3Rs.

CONCLUSIONS

Altogether, these results will showed the complexity of the TME can be recapitulated *in vitro* and to which extent; in addition, they will validate (or not) pre-clinical models of cancer cachexia in terms of muscle impairment, an issue that is particularly important on a clinical point of view. We will design a typology of the hypotheses that are characteristic of the TME-cancer cachexia field based on the degree to which complexity of interactions is involved (synchronically and diachronically) in order to assess their exploratory versus immediately testable features and the adequacy of the associated computation of number of needed animals.

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