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Questions and answers on allogenic mesenchymal stem cell-based products for veterinary use: specific questions on tumorigenicity

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30 Churchill Place ● Canary Wharf ● London E14 5EU ● United Kingdom
Telephone +44 (0)20 3660 6000 **Facsimile** +44 (0)20 3660 5555
Send a question via our website www.ema.europa.eu/contact

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Background

The use of stem cells, in particular mesenchymal stem cells (MSCs), in the veterinary sector is increasing, raising several challenging questions for the end users, manufacturers and authorities. One of the obstacles to using stem cells as the basis for regenerative medicine therapies is the potential capacity of the stem cells to become tumorigenic. Tumorigenicity is defined as the capacity of a cell population inoculated into an animal model to produce a tumour by proliferation at the site of inoculation and/or at a distant site by metastasis. Unique abilities of stem cells to self-renew and differentiate into a variety of cell types are also mechanistically linked to their tumorigenic potential. Although the possibility for stem cells to become tumorigenic is currently perceived as a valid concern in human products, it is not yet clear to which extent this may be significant for the veterinary sector and how this risk could be eliminated, or at least minimized.

While available publications in the human field discuss tumorigenicity of various stem cell types, such as embryonic, mesenchymal, stromal, haematopoietic or induced pluripotent stem cells, it should be emphasized that tumour formation in human patients after MSC administration has not been reported to date. The original observations of tumour formation for isolated MSCs were associated with MSC cultures that were contaminated with tumour cell lines.

To date, it would appear that only MSCs are currently used in the veterinary sector. Given that stem cells in general are suspected to have potential tumorigenic capacities, the use of MSC-based products in the veterinary sector necessitates raising similar questions with regard to their safety profile as for the other stem cell types.

Following a review of the scientific information relating to stem cells, a number of areas have been identified that would benefit from further consideration by relevant experts and, where appropriate, the elaboration of specific guidance in the form of question and answer document (Q&A).

Considering that the scope of this document is restricted to the issue of tumorigenicity, and particular focus will be given to the use of allogeneic MSCs, the questions below are raised in the context of data requirements for a marketing authorisation application for MSCs in the veterinary field, in particular for dogs and horses.

Nine specific questions for further consideration have been identified relating to quality and safety aspects. These questions, together with an answer to each question, are presented below.

1. **Biodistribution: Given the need for biodistribution assays and the anticipated difficulties to achieve them, what advice could be given to facilitate their implementation in the veterinary sector?**

   To date, numerous biodistribution studies have been performed in rodents to assess the fate of human MSCs (hMSCs) or animal MSCs following systemic or local administration. Whatever the model or route of administration, the results showed a poor engraftment of MSCs in the recipients, even more when allogeneic MSCs are transplanted [1]. When systemically infused, MSCs can non-specifically distribute throughout various tissues and organs including lung, liver, kidney and spleen. When administered locally, it is generally accepted that they act through paracrine signalling, while long-term persistence or engraftment of the cells may rarely occur.

   Long-term evaluation of biodistribution has been performed in immunodeficient mice submitted to total body irradiation or local irradiation (abdominal or leg irradiation). Total body irradiation induced an increased hMSC engraftment level compared to non-irradiated mice, while local irradiations increased hMSC engraftment locally in the area of irradiation. In all cases, no tissue abnormality or abnormal hMSCs proliferation was observed at 120 days after irradiation. These

results support the safety of MSCs and showed that MSCs do not undergo transformation and do not promote tumour formation.

It should however be emphasized that evidence is currently insufficient to rule on the relevance of biodistribution results obtained in mouse models, when extrapolated to dogs or horses. Generally, the data produced in xenogeneic or allogenic hosts should be interpreted with care as immune rejection of the administered cells or immune suppression of the host animal are likely to have an impact on the persistence, engraftment and biodistribution of the administered cells [2].

Few studies have been conducted in the dog or horse to analyse the fate of MSCs following local or systemic administration. It is acknowledged that it is experimentally challenging to detect MSCs (in low amounts, if present) in the large organs of these species, where analyses can only be done on biopsy samples that represent a randomly selected piece of a particular organ or tissue. A recent paper describes the evaluation of the fate and distribution of BrdU-labelled autologous bone marrow MSCs after intra-arterial administration in osteonecrosis of the femoral head in dogs [3]. Eight weeks after administration, BrdU-positive cells were unevenly observed in organs by using immunohistochemistry, which included right femoral head, heart, lung, liver, spleen, kidney, gallbladder, small bowel, pancreas, prostate, and testicle. During the whole experiment, all animals survived and no obvious adverse effects were observed. In the horse, the biodistribution of technetium99m-labelled MSCs following different routes was evaluated in animals suffering from tendinopathy [4]. Cells were detected in the lungs most frequently after intravascular administration, although with no adverse effects.

These observations are in accordance with the data in biodistribution assays performed with rodents, showing mainly the persistence of grafted cells in the liver, lungs, kidneys and spleen.

Taking into account the proposed route of administration of the product (for example, an anatomical site from which little biodistribution would be expected to occur), it may not always be necessary to conduct biodistribution assays in the target species for the purpose of supporting an application for a marketing authorisation for MSCs. If there are relevant peer-reviewed bibliographic data available that are possible to extrapolate to the particular product with sound scientific justification, this may support the absence of proprietary biodistribution assays. However, if the route of administration would present a greater potential for biodistribution, consideration should be given to the conduct of such studies, focusing in particular on liver, lung, kidney and spleen, which are considered as the main tissues where MSCs can be trapped.

When MSCs are administered locally, setting up a biodistribution analysis will not be relevant, as cell migration and engraftment in multiple tissues throughout the body is considered marginal [5, 6].

2. Homogeneity of MSCs: Given that homogeneity of any stem cell population is a priori a primary key factor for a sound investigation of its tumorigenic potential, which parameter(s) should to be taken into consideration and which controls could be implemented to ensure getting ultimately homogeneous canine and equine MSCs populations?

While there is a wealth of information on the clinical use and safety of hMSCs indicating that hMSCs are not commonly associated with tumorigenicity, the reports of such events to date seem to be related to contaminations, in particular through cross-contaminations with malignant cell lines as reported previously in literature [7]. However, species differences may occur as exemplified with mice MSCs which have been shown to give rise to tumours *in vivo*. Much less information is available on e.g. canine or equine MSCs.

Homogeneity of the cell population is crucial and highly dependent on the availability of the appropriate molecular and cellular tools. Purification of cell preparations is usually performed with antibody-based devices, like fluorescence-activated cell sorting (FACS) for small scale.

Because homogeneity of MSCs preparations is also largely dependent on the manufacturing process used for their production, it has been suggested to use culture conditions that limit physiological stress and limit their expansion *ex vivo*. Cell modification should be considered and the duration of cell culture and the maximum cumulative population doublings should be specified and validated.

Even if MSCs are a heterogeneous cell population, it is accepted that MSCs isolated from adult tissues (collected after birth) have a limited proliferation capacity *in vitro*.

In 2010, Tarte et al. published the results from a longitudinal study of 20 clinical-grade hMSC preparations obtained using two distinct and well-defined culture conditions and showed that the cells do display some chromosomal abnormalities as detected using conventional karyotype and fluorescent In Situ Hybridization (FISH) analysis. Nonetheless, acquisition of chromosomal abnormality did not appear to confer any selective mitogenic advantage *in vitro* and disappeared rapidly after the second or the third passage [8]. These results have been contradicted by further data published in 2011 [9], showing that MSCs may acquire chromosomal aberrations during *in vitro* expansion. Nonetheless, this work has been challenged by experts as some MSCs evaluated in the work and presenting chromosomal aberrations were derived from foetal liver. In conclusion, the experts argue that genomic stability of cultured adult stem cells and MSCs in particular is robust [10].

As it is too early at this stage to draw definitive conclusions when considering animal MSCs, it is suggested:

- to monitor cell growth beyond the maximal culture time defined for the manufacturing process to detect any genetic instability leading to aberrant cell growth,
- to perform senescence analysis (for instance, senescent cells show common biochemical markers such as expression of an acidic (pH 6) senescence-associated β-galactosidase (SA-β-gal) activity), as aged MSCs could reveal a dramatically reduced biodistribution in both young and aged recipients [11],
- to take account on an ongoing basis of any scientific and technical progress which could improve the homogeneity of the MSC population to be used,
- to achieve batch-to-batch consistency (whatever the degree of homogeneity), for which an acceptable safety profile in the target species was demonstrated,
- to keep cell samples for later analysis.

3. Amplification of MSCs: It is acknowledged that the population doubling level should be limited when stem cells are cultured. Given that amplifications represent an increased risk for inducing or selecting stem cells with potential tumorigenic capacities, what further precautions should be taken during the culture and amplification steps of MSCs to avoid or minimize production or selection of tumorigenic MSCs? Are there culture components or techniques to be avoided?

In general over-expansion of cells should be avoided since high proliferative rate may potentiate the risk of genetic instability. MSCs however seem to be more prone to senescence than to tumorigenicity when cultured for excessive population doublings. Indeed, MSCs are somatic cells with a limited proliferation potential. Experimental data have shown that after 20 to 50 population

doublings, hMSCs undergo replicative senescence, with telomere shortening and increased p16 expression [12]. Although it is expected that human somatic cells have little tumorigenic potential because of their strong inclination to pass into senescence [13], the situation is nonetheless not defined to the same extent for animal MSCs.

MSCs should be very carefully characterised before any use. It is important to validate culture conditions during process development, which avoid the occurrence of genetic instability. Furthermore, cells should be very carefully characterised again after any manufacturing or amplification process. It is important to realise that tests implemented for characterisation during product development are generally more extensive than those used for batch release, in particular because the type of testing performed at batch release may be limited due to the very short shelf life of stem cell products. Moreover, the manufacturing process must consistently yield the same product.

Age of donor is important, as it is known that chromosomal abnormalities accumulate over time. Tissues from newborn tissues represent an attractive source of MSCs regarding this point. Healthy donors should be selected as a chronic inflammatory environment may increase cell susceptibility to chromosomal abnormalities. Donors should be maintained in premises subject to rigorous health control procedures.

There are currently no known culture components or techniques to be avoided, which could represent an increased risk for inducing or selecting stem cells with potential tumorigenic capacities.

4. Amplification of MSCs: Which *in vitro* controls could be implemented on the MSCs at the end of the amplification step to detect tumorigenic MSCs?

Tumorigenic potential of MSCs may be related to the genomic (in)stability of the cells, which may result in aberrant morphology or growth kinetics. Furthermore, there are a number of *in vitro* techniques, which can be used to address cell transformation including growth in soft agar/anchorage-independent growth determination of telomere length and oncogene activation e.g. c-Myc expression. Depending on the anticipated risk of tumorigenicity linked to e.g. extensive handling of MSCs and, consequently, on the level of assurance required, one or more of the techniques mentioned below could be used:

Potential *in vitro* methods to detect genomic instability:

1. There are largely two groups of techniques that are suitable to detect genetic instability of cells: I) conventional karyotyping GTG banding or spectral karyotyping and molecular cytogenetic techniques such as Fluorescent In Situ Hybridization (FISH) and/or Single Nucleotide Polymorphism (SNP) array or II) Comparative Genomic Hybridization (CGH). Conventional karyotyping has poor sensitivity and resolution (5-10 Mb) but is suitable to detect balanced rearrangements and should be used in combination with CGH that has higher sensitivity (≤ 50 kb) but is rather limited in detection of low number of cells with abnormalities (mosaicism) and balanced rearrangements. CGH is not the appropriate method to detect translocations. If aberrations have been detected by karyotyping, further characterization of putative chromosomal aberrations could be continued with immunofluorescence and FISH to detect minor structural abnormalities. Implementation of such techniques might be useful when setting up the production process and are only necessary for batch release when chromosomal abnormalities were found [14, 15].

Potential *in vitro* methods to detect phenotypic and growth abnormalities:

2. Analyze morphology (visual) of the cells to check whether morphology represent homogenous stromal phenotype.

3. Follow growth kinetics of the cells by analyzing aberrant growth kinetics, doubling times and population doublings.

4. Colony formation in soft agar gels or potential for anchorage independent growth. The proliferation of cells in semi-solid media is an established endpoint for clonogenic assays and an indication for transformation. Transformed cells acquire the ability to grow as colonies in semi-solid media (anchorage-independent growth).

Potential *in vitro* methods to detect for a transformation phenotype:

5. c-Myc expression: The proto-oncogene c-Myc encodes a phosphoprotein that exhibits pivotal roles in cell proliferation, where deregulation of c-Myc expression is a contributor to tumorigenesis [16].

6. Telomere length and telomerase activity. The proliferative capacity of human cells is causally linked to the maintenance of telomeres.

5. Genetic stability: Would gross karyotyping be sufficient or would it be necessary or feasible to go into more detailed techniques before engraftment of MSCs into the host in the veterinary sector?

This depends on the anticipated risk of tumorigenicity linked to e.g. extensive handling of MSCs and, consequently, on the level of assurance that is aimed for. The application of karyotyping and supplemental methods (CGH / SNP analysis and FISH) is also explained under Question 4. As stated above, it is important that the production process reproducibly yields the same product. When this is the case, it is reasonable to use less extensive testing for routine batch release than has been used for characterisation during product development if sufficient and consistent data have been presented.

In 2013, a meeting gathering experts in the field of hMSCs was organized to elucidate the risk of potential tumorigenicity related to MSC-based therapies [14]. Regarding the techniques available to assess cytogenetic abnormalities, it was concluded that “conventional karyotyping can be considered a valuable and useful technique to analyse chromosomal stability during preclinical studies”. The experts recommended to analyse 20 metaphases and to exclude cellular batches if 2 identical abnormal metaphases are found (10%). If no chromosomal abnormalities are observed, there should be no need to perform cytogenetic testing on each batch as release criteria.

The occurrence of recurrent abnormalities appears to be mainly related to the manufacturing process more than donor-related. It is then recommended to apply a controlled manufacturing process, avoiding overstressing the cells during their amplification. The number of population doublings should be limited to limit the occurrence of chromosomal abnormalities.

6. Tumorigenesis: Currently, *in vivo* tumorigenicity tests on animals (either on laboratory or on target species) appear to be difficult to implement or give unreliable results. If, however, it is considered feasible to perform *in vivo* tumorigenicity tests on animals, what is the optimal study duration (period of follow-up) to get reliable results? Which parameters and endpoints should be taken into consideration?

Extensive clinical experience with hMSCs demonstrates a good safety profile and negligible risk of tumorigenicity if cell culture and manufacturing process are adequately controlled [14]. Genetic instability that may lead to increased risk of tumorigenicity is predominantly caused by the culture conditions, manipulation of the cells and prolonged cultivation time. Therefore, the key to control

the risk of tumorigenicity of MSCs is to have a well-defined and adequately controlled manufacturing process with appropriate in-process controls and release tests.

In the veterinary sector, it should be emphasized that the classical target animal safety studies will in general not be able to identify any potential tumorigenic risk associated with a product because the timeframe of their implementation is rarely compatible with the potential occurrence of tumours. Possible tumour occurrence may take months or years to appear and may not be suspected by a veterinarian for a given case to be causally linked with treatment; they can in theory appear in various tissues and organs, not systematically at the administration site. Therefore, pharmacovigilance is unlikely to be a useful means of detecting tumours originating from, or caused by, MSC administration.

However, based on an anticipated increased risk of tumour formation due to e.g. extensive manipulation of the MSCs, changes of the culture conditions and/or in case of expected biodistribution, there might be a need for tumorigenicity testing. It is however important to emphasize that, despite the implementation of testing, it will always remain possible that cells migrate to an unwanted location and give rise to a tumour. Rare events are always difficult to detect experimentally. This uncertainty should be addressed through a benefit/risk assessment. For example, if an MSC-based product was available for the treatment of lameness in horses but there was an unquantifiable risk that in the long term there was the potential for the product to be tumorigenic, it may be a case that the benefits outweigh the risks (rather than the horse remaining untreated, a good quality of life for a number of years could be achieved before a tumour may arise).

In conclusion, no tumorigenicity study designs are currently available which will allow to fully exclude the possibility of tumour formation linked to the use of MSCs, but *in vitro* tumorigenicity assays should be implemented to ensure that the manufacturing process is adequately controlled with regards to culture conditions that may increase the risk of tumorigenicity and to get valuable information on the tumorigenic potential of the MSCs to be used. Therefore, it will be necessary to give consideration to incorporating *in vivo* assessment of tumorigenicity. In this context, following approaches are suggested:

- *In vivo* testing of tumorigenicity could be investigated by injections of the cells (as finished product) subcutaneously or intramuscularly in immunodeficient mice (nude, SCID, NOD/SCID/γCnul l, etc…) and monitoring for tumour growth post-injection of MSCs. The principles of such an *in vivo* test are described for example in the WHO Guideline TRS978 [17] (although MSCs do not specifically fall within the scope of this guideline). For MSCs, monitoring for tumour growth 16 weeks post injection for MSCs may be sufficient. Such *in vivo* tests can provide information on the tumorigenic potential of the cells, without taking into consideration the influence of the environment of the cells after administration of the drug product.
- *In vivo* testing of the risk of tumorigenicity of the MSCs in the target species is constrained by the fact that tumour formation, if it were to occur, is anticipated to be a rare event and would not be expected to be temporally associated with treatment. Therefore, concerted effort to conduct appropriate long-term follow-up of treated animals should be made for at least three year post-treatment, as a post-marketing follow-up measure, to allow further refinement of the SPC.

  To be noted that it may be useful to develop specific follow-up measures. For instance, in order to distinguish tumours formed by administered stem cells from tumours developed by other causes, it may be helpful to employ genomic differences between donor and recipient: e.g. in

allogeneic setting when the donor is male and the recipient is female, tumours of allogenic origin can easily be identified by the presence of a Y chromosome.

There is also concern about the ability of MSCs to promote growth of existing or new tumours of other origins. This cannot be addressed by a tumorigenicity study, but the long-term follow-up in the field study or the post-marketing follow-up allows for some assessment of this risk.

7. Tumorigenesis: Are there any options to influence on the pharmaceutical formulation of the product to be injected to minimize the risk of tumour induction?

Pharmaceutical formulation of biologicals is usually minimal and cells would be expected to be suspended in the buffer in which they are cultured or thawed. In general cell products after thawing are quite vulnerable and re-formulation (e.g. to remove the DMSO used for cryopreservation) may do more harm than good.

There are currently no known options with regard to adjusting the pharmaceutical formulation as such to decrease/minimize the tumorigenic risk of the product. Possible avenues are however mooted to reduce the tumorigenic risk, for instance (and if relevant):

- By using osteoinductive agents (Ca-triphosphate) or osteogenic growth factors that would induce differentiation of the adipose derived MSCs and thereby potentially reduces the risk of tumorigenicity of undifferentiated MSCs.
- By limiting the spread or migration of the cells from the site of administration to other organs/tissues, through local administration of the MSCs, or by using hydrogel (which improves the retention of the administered cells at the site of administration).

8. Tumorigenesis: Taking into account that MSC can spontaneously differentiate into fibroblasts producing extracellular matrices, is there a risk that produced material might result in deleterious tissues, especially if administered into an environment where space is limited, e.g.an eye or a myocardium scar?

According to the current level of knowledge on MSCs, it is considered unlikely that MSCs would differentiate into fibroblasts and when this is seen, it is most likely due to contaminations of the cell preparation.

Purity and homogeneity of the cell preparation are crucial and should be adequately controlled.

9. Tumorigenesis: Taking into account that in immune privileged sites even non-transformed MSCs might multiply in an uncontrolled way, is there a risk of any tumour formation by normal healthy cells (non-malignant tumorigenicity)?

Risks of very rare tumour formation can never be entirely excluded, and as stated above will be difficult to observe in a (reasonably sized) experiment. Furthermore, hMSCs seem to have a good safety track record.

Residual risks of the magnitude intended here, should probably be dealt with in the benefit/risk evaluation.

Main abbreviations used in the document

CGH: comparative genomic hybridization

SNP: single nucleotide polymorphism

hMSCs: human mesenchymal stem cells

FISH: fluorescence in situ hybridization

MSCs: mesenchymal stem cells

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