Research in Veterinary Science 117 (2018) 104–110

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Gold nanoparticles enhance radiation sensitization and suppress colony formation in a feline injection site sarcoma cell line, *in vitro*

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ARTICLE INFO

Keywords:
Injection site sarcoma
Gold nanoparticles
Radiation
In vitro

ABSTRACT

Injection Site Sarcomas (ISS) are highly invasive feline malignant tumors that are frequently associated with routine vaccination. Current treatment modalities include chemotherapy, radiation, and radical surgery. ISS have been shown to be one of the most treatment resistant of feline cancers with high rates of recurrence. Previous studies have shown that gold and other high atomic number nanoparticles have the ability to increase the dose of radiation deposited into tissue by generating secondary electrons. The focus of the current study was to assess the effects of gold nanoparticles (AuNP) on ISS cytotoxicity and colony formation both as a standalone treatment and in combination with electron beam radiation. Cells from an established ISS cell line were co-cultured with 15 nm AuNP at 0.0, 0.25, 0.5, 1.0, 2.0 and 4.0 mM. AuNP cytotoxicity was evaluated by assessing changes in cellularity, cell proliferation, cell cycle and viability/apoptosis/necrosis. The radiosensitizing potential of AuNP on ISS replication was assessed by the clonogenic assay. AuNP were found to significantly decrease cellular proliferation. However, the acute viability and cell cycle of ISS was not significantly altered. Interestingly, AuNP alone were shown to significantly impair colony formation. In the presence of 9 MeV electron radiation, AuNP numerically decreased colony formation in ISS cells compared to cells treated with radiation only. AuNP may have efficacy as a long term therapeutic agent for decreasing ISS growth.

1. Introduction

Injection site sarcoma (ISS) is an aggressive tumor that occurs in cats. ISS is frequently associated with rabies and leukemia vaccines occurring a varying duration of time after immunization (Kass et al., 1993). At the cellular level, reactive fibroblasts along the periphery of the nodule that forms at the site of injection undergo malignant transformation, although the exact mechanism still remains unknown (McEntee and Page, 2001; Wilcock et al., 2012). The degree of inflammation is influenced by variation in the formula of the vaccine and the cat's genetically conditioned response. The resulting inflammation can then lead to neoplastic transformation and the formation of sarcoma nodules (Macy and Hendrick, 1996; Cohen et al., 2001). These nodules may be detected between two months and ten years after vaccination (Saba, 2017). These tumors are often very invasive and have a high postoperative recurrence rate plus are usually surrounded and infiltrated by lymphocytes and macrophages (Hendrick and Brooks, 1994). The estimated rate of metastasis of these tumors is 10–25% with lungs and regional lymph nodes being common metastatic sites (Cronin et al., 1998; Romanelli et al., 2008; Saba, 2017).

Vaccine associated sarcomas were first reported in 1991 following legislation requiring the use of killed rabies vaccine in cats in the United States. The incidence of ISS is between 1 in 1000 and 1 in 10,000 vaccinated cats. Despite the relatively low risk, around 2000 ISS are reported each year in the United States (Wilcock et al., 2012). Current treatment protocols for vaccine associated sarcoma focus on radical surgical excision of the tumor. However, the tumor reoccurrence rate of 6 months post-surgery is still high at 70% (Hartmann et al., 2015) with the median survival time for cats treated with surgery alone at 576 days (Hershey et al., 2000). If surgery is performed early with radical excision, it is possible for the treatment to be curative (Phelps et al. 2011). Unfortunately, complete resection is often difficult due to the invasive nature of the fibrosarcoma (Kobayashi et al., 2002). Radiotherapy is used to treat microscopic remains of the disease and may improve the time to first event following surgery, but the approach requires a large radiation field limiting the dose that can be safely delivered. When

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https://doi.org/10.1016/j.rvsc.2017.11.018
Received 27 June 2017; Received in revised form 4 October 2017; Accepted 26 November 2017

radiation therapy is combined with surgery, tumor recurrence can still occur regardless of whether tumor cells are absent post resection, indicating a continuing need for more aggressive therapy (Cohen et al., 2001, Eckstein et al., 2009). A safe and effective radiation sensitizing agent that targets tumor cells and avoids normal tissue could improve the efficacy of radiotherapy and local tumor control.

Nanoparticles are defined as a material that is < 100 nm, which has led to the creation of the term, "zero-dimensional materials" that can serve as building blocks to create mechanisms for medicinal therapy and increased ability to visualize tissues (Murthy, 2007). In recent years, the therapeutic application of nanoparticles has been studied due to their extremely small size, variability in composition and structure. Nanoparticles have a wide variety of compositions, consisting of inorganic, organic polymers, or a combination that allows for the desired function of the nanoparticle. Both types of nanoparticles have the ability to be formulated to carry a specific drug or be targeted to a particular tissue (Murthy, 2007). There are a variety of factors that determine how long a nanoparticle is able to exist in the body, such as size and charge of the particle (Cao-milán and Liz-marzán, 2014). Inorganic nanoparticles such as silver, gold, and silicone, are often used when tissues or cells need to be visualized using optical or nuclear imaging technology. Continuing technological advances have allowed for improvement in synthesis, testing, and biomedical uses of nanoparticles. Gold Nanoparticles (AuNP) can be bound non-covalently to chemical agents in order to increase the bioavailability of the drug. A study by Wójcik et al. (2015), showed that AuNP bound to the chemotherapy drug doxorubicin could elicit a higher cytotoxic response in ISS cells as well as decrease resistance to doxorubicin (Wójcik et al., 2015).

Gold nanoparticles have also been shown to increase radiosensitivity in other mammalian tumor lines and thus, may have efficacy in the treatment of ISS (Kong et al., 2008; Rahman et al., 2009; Wolfe et al., 2015). AuNP can be preferentially targeted to cancer cells and have been shown to increase the radiosensitivity of cells due to their photoelectric absorption of photons. Once internalized AuNP encounter a photoelectron field, they can produce additional low energy, short range, secondary electrons inside the cell (Hainfeld et al., 2008). Currently, it is believed that high atomic number materials (Z), such as gold, increase the amount of localized energy initially provided by radiation therapy through excitation of delocalized electrons in the metal, called plasmons. AuNP may also increase the bystander effect, whereby non-irradiated cells exhibit characteristics of irradiated cells through reactive oxygen and nitrogen species released from nearby radiated cells (Haume et al., 2016). The increased energy within the cell may result in the formation of reactive oxygen species and induce oxidative stress (Butterworth et al., 2012). Prior studies on radiosensitization by gold nanoparticles have utilized numerous doses and types of ionizing radiation, kilovoltage and megavoltage beams. Although kilovoltage X-rays have been shown in previous studies to have a dose enhancing effect with gold nanoparticles, a study of veterinary radiation facilities by Farrelly and Mcentee (2014) reported that all facilities surveyed had a linear accelerator and 79% had electron capability. The greatest degree of dose enhancement and cellular uptake in mammalian cells has been shown with 50 nm gold nanoparticles, although a number of cell lines have been reported to take up a variety of sized nanoparticles. Dose enhancement also increases with the concentration of gold inside the cell up to 0.5 mM (Rahman et al., 2009). It was also observed that the increase in radiation was accompanied by an increase in double strand DNA breaks within the cell. There are a number of factors that influence how AuNP are internalized by cells. One challenge working with AuNP is that they can aggregate once introduced into an aqueous environment. This can be prevented by using polyethylene glycol or encapsulating the AuNP within a lysosome, which can also increase circulation time within the body (Yildirimer et al., 2011). Once internalized, the cytotoxity of AuNP is largely dependent on nanoparticle size. AuNP within the range of 10-15 nm have been shown to be relatively non-toxic, *in vitro*. However, it has been shown that AuNP alone may increase reactive oxygen species in cells (Haume et al., 2016). Several *in vivo* studies have yielded mixed results regarding the systemic toxicity of AuNP, ranging from severe to negligible toxic effects. However, due to the lack of standard research parameters, AuNP's toxicity has not been firmly established (Butterworth et al., 2012, Khlebtsov and Dykman, 2011).

The focus of the current study was to assess the potential cytotoxic effects of AuNPs alone or when combined with radiation, in ISS. Changes in cellularity (cell recovery), cell viability, cell cycle, and degree of apoptosis/necrosis were evaluated, *in vitro*. The ability of AuNP-cultured cell clones to establish new colonies post electron beam radiation was also assessed *via* clonogenic assays.

2. Materials and methods

2.1. Cell culture technique

Cells from a previously established ISS cell line (JB, passages #12–20) were cultured in complete DMEM (10% fetal bovine serum (FBS, Atlanta Biologicals, Flowery Branch, GA), 1% antibiotics (100 units penicillin/mL and 100 µg streptomycin/mL, Mediatech, INC, (Manassas, VA) at 37 °C in an atmosphere of 5% CO_2. Cells were seeded at 150,000 cells/flask grown to 80–85% confluence in T25 flasks (Thermo Scientific, Roskilde, Denmark) and passaged every 5–7 days using 0.5 mL of trypsin. Cells were then centrifuged at 244 × *g* for 7 min at 23 °C and enumerated with a Nexcellom Auto T-4 cell counter (Lawrence, MA).

2.2. Preparation of gold nanoparticles

Gold nanospheres (15 nm) were purchased from Aurovist (Yapank, NY). These nanoparticles were sterilized by the manufacture and supplied dissolved in 0.2 mL PBS after being filtered through 0.22 µm bacterial filter. Nanoparticles were diluted in complete DMEM to desired concentrations.

2.3. Minimum concentration inhibition assay

Cells were cultured in Costar 96 well flat bottom tissue treated cell culture plate (Costar, Corning, New York) at 5000 cells volume per well. Cells were allowed to adhere for 24 h prior to addition of nanoparticles at which time they were 25% confluent. Cells were then treated with different concentrations of AuNP (0.0, 0.125, 0.25, 0.5, 1.0, 2.0 mM) in 7 mL of complete media. Cells were incubated an additional 64 h, then 20 µL of Alamar Blue was added to each well and incubated an additional 8 h. Cells were about 85–90% confluent at the time proliferation was determined. Proliferation was measured by determining the specific absorbance of each well at 570–600 nm using a BioTek Synergy 4 (Winooski, Vermont) and Gen5 software. Results were enumerated as Δ optical density units then converted to % viability based on growth of cells + media alone being 100% to define the minimum concentration inhibition (MCI) by AuNP exposure. MCI values were reported as average of three replicate experiments.

2.4. Cytologic analysis

Cells were grown on Costar 6-well Tissue Culture Treated Plates. Different concentrations of AuNP (0.0, 0.25, 0.5, 1.0, 2.0 mM) in complete DMEM were added to cells and incubated at 37 °C in an atmosphere of 5% CO_2. At 72 h post nanoparticle culture, cells were detached, enumerated using Nexcellom Auto T-4 cell counter and diluted to 1.5×10^5 cells/100 µL. Cells were suspended in PBS, placed in Wescor Aerospray 7150 Hematology Slide-Stainer-Cytocentrifuge (Logan, UT) and centrifuged at 33.5 × *g* for 3 min. Slides were then allowed to dry for 24 h and then stained with Wright-Giemsa stain